THE EFFECTS OF FLUOXETINE AND QUETIAPINE ON THE PROLIFERATION AND DIFFERENTIATION OF, AND GDNF RELEASE FROM, C6 CELLS

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By

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Abstract

According to the literature, there is a decrease in glial cell number or hypofunction of glial cells in depression. It was also found that both antidepressants and atypical antipsychotics might target glial cells, and that they increase the release of glial-cell-line-derived neurotrophic factor (GDNF) from C6 rat glioma cells (C6 cells). In this project, C6 cells were used as a model for glial cells to investigate the effects of fluoxetine and quetiapine on proliferation and differentiation, and to investigate their effects on the release of GDNF. A combination of quetiapine and fluoxetine was used to study their potential synergistic effect on the release of GDNF from C6 cells.

C6 cells were treated with different concentrations of fluoxetine and quetiapine in both normal and serum starvation culture conditions. Under the serum present condition, fluoxetine (25 μM) decreased the number of C6 cells from 24 to 48 h, while quetiapine (25 μM) decreased the cell number only at 48h. Under serum starvation, it was found that fluoxetine (12.5 μM) increased the number of C6 cells from 24 to 48 h treatment; in contrast, quetiapine (25 μM) decreased the number of C6 cells after 48 h treatment. Both fluoxetine and quetiapine inhibited the proliferation of C6 cells under normal and serum starvation conditions. Fluoxetine (12.5 μM) decreased C6 cell death, while quetiapine had no significant effect. Fluoxetine, but not quetiapine, changed the morphology of C6 cells and increased the level of glial fibrillary acidic protein (GFAP), an astrocyte marker. Both fluoxetine (12.5, 25 μM) and quetiapine (25

μM) increased the release of GDNF from C6 cells, and an apparent additive effect was found between quetiapine and fluoxetine in the modulation of release of GDNF from these cells.

It was concluded that:

1. High concentration (25 μM) of fluoxetine and quetiapine decreased the number of C6 cells under the serum present condition and both drugs inhibited the proliferation of C6 cells.

2. Fluoxetine had a protective effect on the C6 cells under serum starvation, and affected the differentiation of C6 cells; this implies that fluoxetine may protect glial cells in vivo and affect their differentiation.

3. A high concentration of quetiapine decreased the number of C6 cells and inhibited the proliferation under serum starvation; even though it increased the release of GDNF from C6 cells as did fluoxetine.

4. Both quetiapine and fluoxetine increased the release of GDNF from C6 cells under serum starvation. The combination of quetiapine and fluoxetine had an apparent additive effect in the modulation of GDNF release.

5. These effects on proliferation & GDNF release may underlie the benefit observed with these drugs in treating depression and schizophrenia.

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List of Abbreviations

Artemin	ARTN
Brain-derived neurotrophic factor	BDNF
cAMP response element binding protein	CREB
Central nervous system	CNS
Dulbecco's modified Eagle's medium	DMEM
Dopamine	DA
Extrapyramidal symptoms	EPS
Enzyme-Linked Immunosorbent Assay	ELISA
Glial fibrillary acidic protein	GFAP
Glial-cell-line-derived neurotrophic factor	GDNF
Glial-cell-line-derived neurotrophic factor (GDNF) family ligands	GFL
Monoamine oxidase inhibitor	MAOI
Mitogen activated protein kinase	MAPK
Nonrepinephrine reuptake inhibitors	NRI
Neurturin	NRTN
Norepinephrine	NE
N-methyl-D-aspartate	NMDA
Parkinson's disease	PD
Persephin	PSPN
Prolactin	PRL
Receptor tyrosine kinase	RTK
Selective serotonin re-uptake inhibitors	SSRI
Serotonin	5-HT
Transforming growth factor	TGF
Tricyclic antidepressants	TCA
Tardive dyskinesia	TD

1 Introduction

1.1 The monoamine theory of depression

Depression is a major psychiatric disorder that causes a great economic and social burden. The underlying mechanisms of depression and the mechanisms of action of antidepressant drugs are still not clear. Previous studies of depression and mechanisms of action of antidepressants focused on the monoamine systems (Heninger et al., 1996), that is because almost all of the antidepressants can enhance the functions of noradrenalin (NE) or serotonin (5-HT). For example, amitriptyline, a tricyclic antidepressant, can increase the concentration of NE in the synapse; Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), can promote the function of serotonin; Tricyclic antidepressants (TCAs), including imipramine and amitryptyline, which became available in the 1950s, can increase the synaptic concentration of norepinephrine by blocking its re-uptake in brain tissue (Axelrod, 1981). The effects of TCAs, and the fact that reserpine can cause depression by depleting monoamine neurotransmitters NE, dopamine (DA) and 5-HT, formed the basis of the monoamine hypothesis of depression, which suggested that depression was caused by the deficiency of monoamines (Schildkraut, 1965).

The monoamine hypothesis, however, cannot explain: (1) the delayed onset of antidepressants efficacy, (2) why some patients with depression do not respond to antidepressants, and (3) why the depletion of monoamines in healthy volunteers does not induce depressive symptoms (Vaidya and Duman, 2001).

So the overall mechanism of depression and the mechanism of action of antidepressants may go beyond changes in monoamine levels.

1.2 The neurogenesis theory of depression

1.2.1 The roles of CREB in depression

It was reported that the cAMP response element binding protein (CREB) and neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), might be involved in the mechanisms of action of antidepressants and depression (Duman, 2002).

CREB is a key component of the cAMP-CREB transduction cascade; it also serves as a central integrator of signaling for many extracellular stimuli. The cAMP-CREB transduction cascade is regulated by both the serotonin and NE neurotransmitters, both of which are affected by antidepressants (Duman, 2002).

CREB expression is altered in the brain of patients with depression; it was found that the levels of CREB are decreased in the temporal cortex of patients with depression who were not receiving medication at the time of death (Dowlatshahi et al., 1998).

Antidepressants and mood stabilizers can regulate the expression of CREB. It was reported that the level of CREB protein was increased in the cerebral cortex of patients treated with an antidepressant at the time of, or for a period shortly before, death (Dowlatshahi et al., 1998). Antidepressants and

long-term administration of mood stabilizers, such as lithium and valproate, can increase the phosphorylation of CREB (Nibuya et al., 1996; Manji et al., 2000; Thome et al., 2000).

CREB has an antidepressant-like effect. It was demonstrated that overexpression of CREB in the dentate gyrus of the hippocampus had antidepressant-like effects in the forced-swim and learned-helplessness models of depression (Duman, 2002).

The above evidence supports CREB's involvement in the pathology of depression and the mechanism of action of antidepressants.

1.2.2 The roles of BDNF in depression

BDNF levels were reduced in animal models of depression; this reduction was reversed by antidepressants. For example, immobilization stress can induce a down-regulation of hippocampal BDNF, which can be blocked by antidepressant treatment (Nibuya et al., 1995). This decrease in BDNF levels can be reversed by repeated antidepressant administration, including NE or 5-HT selective reuptake inhibitors (Nibuya et al., 1995; Russo-Neustadt et al., 1999). Repeated restraint stress also reduced the BDNF levels and hippocampal cell proliferation; antidepressants including fluoxetine and venlafaxine reversed the reduction of both BDNF and hippocampal cell proliferation (Haynes et al., 2004; Xu et al., 2004; Sairanen et al., 2005).

Like CREB, BDNF has an antidepressant-like effect. One study found

that infusion of BDNF for 7 days via osmotic minipump had an antidepressant-like effect in the forced-swim and learned-helplessness models of depression (Siuciak et al., 1997).

1.2.3 Neurogenesis in depression

The notion that the adult nervous system can generate new neurons, so-called adult neurogenesis, has been supported by many studies (Gould et al., 1998; Lledo et al., 2006). Adult neurogenesis occurs in some specific areas; for example, it was reported that hippocampus has the ability to generate neurons throughout life (Eriksson et al., 1998).

Adult neurogenesis might be decreased in depression. It was found that repeated stress induces atrophy, or even death in severe cases, of CA3 pyramidal neurons in animal models of depression, and it was noted that this stress resulted in decreased neurogenesis in the hippocampus (Gould et al., 1998; McEwen, 1999). Neuroimaging studies found that the size of the hippocampus of patients with depression is reduced (Bremner et al., 2000); it was also reported that the volume of subgenual prefrontal cortex was decreased (Drevets et al., 1997). A decrease in the number of neurons was also reported in patients with depression (Rajkowska et al., 1999). These studies indicate that there might be a decrease in adult neurogenesis in both depressed patients and in animal models of depression.

Antidepressants may increase adult neurogenesis in depression. It has

been reported that repeated antidepressant administration significantly increased the rate of cell proliferation in the hippocampus of normal rats. Antidepressants used in this study included NE or 5-HT selective reuptake inhibitors (Malberg J et al., 2000). Fluoxetine was also reported to block the down-regulation of neurogenesis induced by inescapable stress (Malberg and Duman, 2003). It was also reported that hippocampal neurogenesis was increased by long-term lithium treatment (Chen et al., 2000). All of these findings support the notion that antidepressants may increase neurogenesis or prevent the decrease in neurogenesis observed in depression.

1.2.4 CREB and BDNF mediate neurogenesis

It has been speculated that the effect of antidepressants and mood stabilizing drugs on neurogenesis was mediated by CREB and BDNF (Vaidya and Duman, 2001). On the basis of the above findings, the role of CREB in the regulation of neurogenesis was examined. Transgenic overexpression of dominant negative CREB, which can cause a decrease in normal CREB, decreased neurogenesis in the hippocampus, and this indicated that CREB might be involved in the neurogenesis in the hippocampus (Nakagawa et al., 2000). Similarly, BDNF increases neurogenesis in hippocampus. Intrahippocampal BDNF infusion was found to increase neurogenesis (Scharfman et al., 2005).

The neurogenesis theory of depression hypothesized that stresses

induce a decrease in neurogenesis in hippocampus, while antidepressants can reverse the decreased neurogenesis, thereby promoting recovery from depression (Jacobs et al., 2000).

This hypothesis seems interesting and reasonable, but it does not explain some phenomena in depression: in particular, hypofunction of glial cells, especially astrocytes (Cotter et al., 2001).

1.3 The functions of glial cells

"If the functional unit of the brain is not the neuron but rather the neuron-glial complex, then both neuronal and glial cells could be involved in mental diseases" (Bogerts et al., 1983). Berland Bogerts said these words to describe that glial cells have been viewed as having a passive role in the central nervous system (CNS), and their functions in the CNS may be underappreciated. There are three types of glial cell in the CNS: astrocytes, oligodendrocytes and microglia. The important functions of astrocytes include regulation of synaptic function, transmitter levels and neuronal metabolism (Cotter et al., 2001). Some evidence indicated that astrocytes may be involved in the pathology of depression (Cotter et al., 2001), so I will focus on the functions of astrocytes.

Astrocytes are rich in glucose transporters, and the energy released by glycolytic processing is required for the conversion of glutamate to glutamine in astrocytes (Araque et al., 1999). Astrocytes can release lactate which can be taken up by neurons; lactate is the major energy source for nerve terminals

(Coyle and Schwarcz, 2000).

Astrocytes can promote synapse formation in vitro, and also are involved in the development and remodeling of synaptic connections (Pfrieger and Barres, 1997). The transporters in astrocytes can modulate the concentration of neurotransmitters in the synapse. The glutamate uptake system in astrocytes can regulate the duration of the excitatory current. The glutamate taken up by astrocytes can be converted into glutamine and transported back to the neuron for reuse in neurons (Tanaka et al., 1997).

Astrocytes are involved in the regulation of the N-methyl-D-aspartate (NMDA) receptor (Enomoto et al., 1998; Wolosker et al., 1999). Functional activity of NMDA receptors requires both glutamate binding and the binding of an endogenous co-agonist, glycine. D-serine is an endogenous modulator of the glycine site of NMDA receptors. The serine racemase, an enzyme from astrocytes, can convert L-serine to D-serine on NMDA receptor, thereby modulating the function of NMDA receptors.

The most important functions of astrocytes related to depression may be due to their neurotrophic functions. Astrocytes can release many kinds of neurotrophic factors including BDNF, glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor (FGF-2), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5) (Cotter et al., 2001; Tokumine et al., 2003; Chadi and Gomide, 2004). Much evidence indicates that BDNF is involved in the pathogenesis of depression and the mechanism of action of antidepressants

(see Section 1.2.2).

1.4 The role of glial cells in depression

There is some evidence that supports the notion that glial cells might be involved in the cellular biology of depression. Many studies about the cellular biology of depression have focused on postmortem research. Glial cell number was reduced in certain brain areas such as prefrontal cortex, cerebellum, amygdala, and hippocampus (Ongur et al., 1998; Chen et al., 2000; Bowley et al., 2002; Cotter et al., 2002). Some studies found decreased neuronal size or neuronal number in different areas (Rajkowska et al., 1999; Cotter et al., 2002); however, the demonstration of a decrease in the number of neurons was not consistent, with some studies not finding any reduction at all (Bowley et al., 2002).

Using animal models of depression, it has been shown that stress can decrease cell proliferation in hippocampus (Cotter et al., 2002; Xu et al., 2004). Most of these studies used BrdU to label newborn cells, and thus, could not differentiate between glial cells and neurons. It is possible that the decreased cell proliferation observed in these studies was due to glial cells rather than neurons.

None of these studies can exclude the possibility that the effect of antidepressants on neurons is preceded by an effect on glial cells, or mediated through glial cells. A reduction in the number of Glial fibrillary acidic protein

(GFAP) labeled cells in postmortem brains of depressed patients has been found, therefore, it was concluded that the reduction in glial cells was due to astrocytes (Fatemi et al 2004; Johnston-Wilson et al 2000; Miguel-Hidalgo et al 2000; Si et al 2004). Only one paper found that the glial cell reduction in the amygdala was due to oligodendrocytes; this study used human leukocyte antigen to label oligodendrocytes (Hamidi et al., 2004). One paper found a reduction in GFAP labeling in younger major depressive disorder (MDD) patients (Rajkowska et al., 1999). The GFAP level can reflect not only the actual number of astrocytes, but it also is indicative of the activation of astrocytes, so one cannot exclude the possibilities that patients with depression have a decreased number of astrocytes and/or a hypofunction of astrocytes. It is not clear whether the decreased level of GFAP and the decreased number of glial cells was caused by depression or antidepressant drug treatments, because most of the postmortem studies did not list patient history or whether patients were untreated and /or drug-free prior to death.

The above evidence supports that glial cells and astrocytes in particular may be involved in the mechanism of depression. A hypofunction of glial cells in depression exists.

1.5 Antipsychotics and their use in depression

Antipsychotics are classified as typical or atypical according to their pharmacological characteristics and clinical profiles. Typical antipsychotics

generally refer to the older antipsychotics, such as chlorpromazine and haloperidol, which have a higher chance of causing extrapyramidal symptoms (EPS) and tardive dyskinesia (TD), and which increase prolactin (PRL) levels (Remington and Kapur, 2000). Clozapine is the prototype of an "atypical" antipsychotic, which has a lower incidence of EPS and TD, and has no effect on PRL (Remington and Kapur, 2000). Other atypical antipsychotics include risperidone, olanzapine and quetiapine, which share similar clinical characteristics with clozapine.

The pharmacological properties of atypical and typical antipsychotics are quite different. Atypical antipsychotics are antagonists of 5-HT2 and D2 receptors with a higher ratio of 5-HT2/D2 receptor affinity, which means that atypical antipsychotics have a relatively lower D2 receptor binding capability and a higher 5-HT2A receptor binding capability (Ananth et al., 2001; Factor, 2002). This is a simplification of the mechanisms of action of antipsychotics, because antipsychotics can affect many other receptors such as α1-adrenergic, α2-adrenergic, histamine H1 and muscarinic receptors (Richelson and Souder, 2000).

The mechanisms of action of antipsychotics drugs can go beyond the receptor level. Gene expression and neurotrophic factors may be involved. Both typical and atypical antipsychotics drugs can induce the expression of the immediate-early gene c-fos in various areas of the brain (Dragunow et al., 1990; Nguyen et al., 1992; Bremner et al., 2000; Ananth et al., 2001), while quetiapine

increased FGF-2 and BDNF expression in the hippocampus of animals treated with the NMDA antagonist MK-801 (Fumagalli et al., 2004). These findings suggest the involvement of neurotrophic factors in the mechanism of antipsychotic drugs.

Neuroprotection might be involved in the mechanisms of action of atypical antipsychotic drugs. First, atypical antipsychotic drugs are neuroprotective (Shao et al., 2006). It has been reported that atypical antipsychotic drugs including quetiapine, clozapine and risperidone protected cultured PC12 cells from cell death caused by serum withdrawal (Bai et al., 2002). Olanzapine, another atypical antipsychotic drug, also protects PC12 from death caused by H_2O_2 or β-amyloid peptide treatment (Wei et al., 2003). Second, the atypical antipsychotic drug, quetiapine, increased BDNF mRNA expression in CA1, CA3, and dentate gyrus regions of the rat hippocampus (Bai et al., 2003). The increased level of BDNF might be neuroprotective, because BDNF plays an important role in neuronal cell survival, differentiation, and neuronal connectivity. Some antipsychotic drugs were also found to stimulate the release of GDNF (Shao et al., 2006) (discussed in detail in section 1.8.4.6). It was concluded that the release of GDNF from glial cells might protect neurons from degeneration associated with diseases such as schizophrenia or Alzheimer's disease (Shao et al., 2006).

Antipsychotics, especially atypically antipsychotic drugs, are often used in the treatment of psychosis in depression (Shelton 2006). It was found that

augmentation of antidepressants with atypical antipsychotics can be used in the treatment of refractory depression and anxiety (Blier and Szabo, 2005; Nemeroff, 2005). These clinical observations suggest a synergistic effect of antipsychotics on antidepressant action.

1.6 The effects of antidepressants and antipsychotics on glial cells

Lithium, a mood stabilizer used especially in bipolar depression, can induce gliosis in rats, and increases GFAP levels in the hippocampus (Rocha and Rodnight, 1994). Lithium and valproate also attenuate or reverse the decrease in glial numbers in mood disorders (Bowley et al., 2002). Recently, it was shown that chronic treatment with the antidepressant fluoxetine or the antipsychotic olanzapine can increase the proliferation of glia, but not neuronal cells, in prelimbic cortex, and can increase neuronal proliferation in dentate gyrus. This study used colabeling with a neuronal marker (NeuN) and an astrocyte marker (S100 protein) to label neurons and astrocytes, respectively (Kodama et al., 2004). It was also found that fluoxetine can increase the content of S-100 protein in hippocampus, and because S-100 is a marker for astrocytes, it was concluded that maybe fluoxetine can increase glial cell proliferation in the hippocampus (Manev et al., 2001) . Considering the fact that there might be a hypofunction of glial cells in depression (see section 1.4), this suggests that these drugs may target glial cells and reverse the hypofunction of glial cells in depression.

Both typical (haloperidol, chlorpromazine) and atypical (clozapine, olanzapine, risperidone) antipsychotics can elevate glial cell density and cortical thickness in prefrontal areas in monkeys, suggesting that glial proliferation may be a part of the mechanism that can reverse the imbalance of transmitters in schizophrenia and in other psychotic illnesses (Selemon et al., 1999).

The above evidence suggests that antidepressants, mood stabilizing drugs and antipsychotic drugs might increase the number of glial cells. Considering the fact that there might be a hypofunction of glial cells in depression, it is possible that both antidepressants and antipsychotics may share a similar mechanism by acting on glial cells in the treatment of depression.

1.7 C6 cells

The C6 cell line, a glial cell strain, was cloned from a rat glial tumor induced by N-nitrosomethylurea (Benda et al., 1968). Its morphology is similar to fibroblasts. Because it is similar to normal glial cells, the C6 cell line is often used as a model for glial cell studies (Yoshimura et al., 1997).

It is well known that C6 cells have progenitor cell properties and that they can differentiate toward astrocytic phenotypes. Treatment of C6 cells with cAMP can induce them to differentiate into astrocytes. Differentiated C6 cells express high levels of GFAP and S-100 protein (Zimmer and Van Eldik, 1989), both of which are specific markers of astrocytes. The differentiation of C6 cells is accompanied by a morphological change, from a bipolar to a multipolar shape,

although the degree of morphological change of C6 cells after differentiation was different between different laboratories (Zimmer and Van Eldik, 1989). It was found that after differentiation the morphology of C6 cells changed from a flat shape to spindle-shaped with processes (Takanaga et al., 2004).

C6 cells express very high levels of GDNF (2,837±813 pg/g protein). Human glioma also has a very high GDNF content (937±140 pg GDNF/g tissue), while the GDNF levels in normal human and rat brain are significantly lower (less than 150 pg/g tissue) (Wiesenhofer et al., 2000).

C6 cells have often been used for the study of GDNF release induced by various biological factors, antidepressants and antipsychotics drugs (see section 1.8.4). In the current project, they were used as a model for the glial cells and for the study of GDNF release.

1.8 GDNF

1.8.1 Background

Much evidence supports that BDNF might be involved in depression (see section 1.2.2). GDNF is also a trophic factor that has widespread functions (in detail in section 1.8.3). It has also been reported that GDNF can be regulated by antidepressants and antipsychotics (Hisaoka et al., 2001; Shao et al 2006). These studies indicated that modulating the release of GDNF might be a possible mechanism underlying the actions of antidepressants and antipsychotic drugs.

Neurotrophic factors are vital for many aspects of neuronal function, such as maintaining the number of neurons, neurite branching and synaptogenesis, adult synaptic plasticity and maturation of electrophysiological properties (Sariola and Saarma, 2003). Neurotrophic factors are classified into neurotrophins, neurokines and GDNF family ligands (GFLs).

GFLs include GDNF, artemin (ARTN), neurturin (NRTN) and persephin (PSPN). They are involved in the support of several neuronal populations in the central nervous system such as midbrain dopamine neurons and motor neurons. GDNF, NRTN and ARTN are also involved in the survival and the differentiation of peripheral neurons such as sympathetic, parasympathetic, sensory and enteric neurons (Manie et al., 2001).

GDNF was first identified in 1993 as a growth factor capable of promoting the survival of the embryonic dopaminergic neurons of the midbrain (Siuciak et al., 1997). Because the members of the GDNF family have seven cysteine residues in the same relative spacing as some members of the transforming growth factor TGF-β superfamily, they are also members of the transforming growth factor (TGF) -β superfamily. The conformation of the GDNF family members are very similar to the two structurally characterized members of the TGF-β superfamily, TGF-β2 and bone morphogenetic protein-7 (BMP-7), although they share less than 20% amino-acid sequence homology with other members of the TGF-β superfamily. Even within the GDNF family members, the amino-acid sequence homology is only between 40 and 50% (Eigenbrot and

Gerber, 1997; Saarma, 2000).

1.8.2 Signaling of the GDNF family members

Although GDNF members are members of the TGF-β superfamily, their signaling pathways are quite different. Unlike other members of the TGF-β superfamily, which signal through receptor serine/threonine kinases (Saarma, 2000), GDNF members activate the receptor tyrosine kinase (RTK) Ret (Treanor et al., 1996; Trupp et al., 1996). GDNF members do not bind directly to Ret; they bind to GDNF family receptors (GFRα), which are bound to the plasma membrane by a glycosylphosphatidylinositol (GPI) (see Fig 1.1).

The ligand-binding specificity of GFLs depends on the individual GFRα proteins. GDNF, NRTN, ARTN and PSPN specifically bind to GFRα1, GFRα2, GFRα3 and GFRα4, respectively (Airaksinen and Saarma, 2002; Sariola and Saarma, 2003). A homodimer of GFLs first binds to the specific GFRα to form a complex. The complex recruits two molecules of Ret (coreceptor for GDNF) to form a bigger complex; the formation of the latter complex induces transphosphorylation of specific tyrosine residues in their tyrosine kinase domains and, thereafter, intracellular signaling (Airaksinen and Saarma, 2002). (see Fig1.1)

Like other receptor tyrosine kinases, the tyrosine residues in the intracellular domain of activated Ret work as binding sites for different intracellular signaling proteins in different cells (Schlessinger, 2000). At least

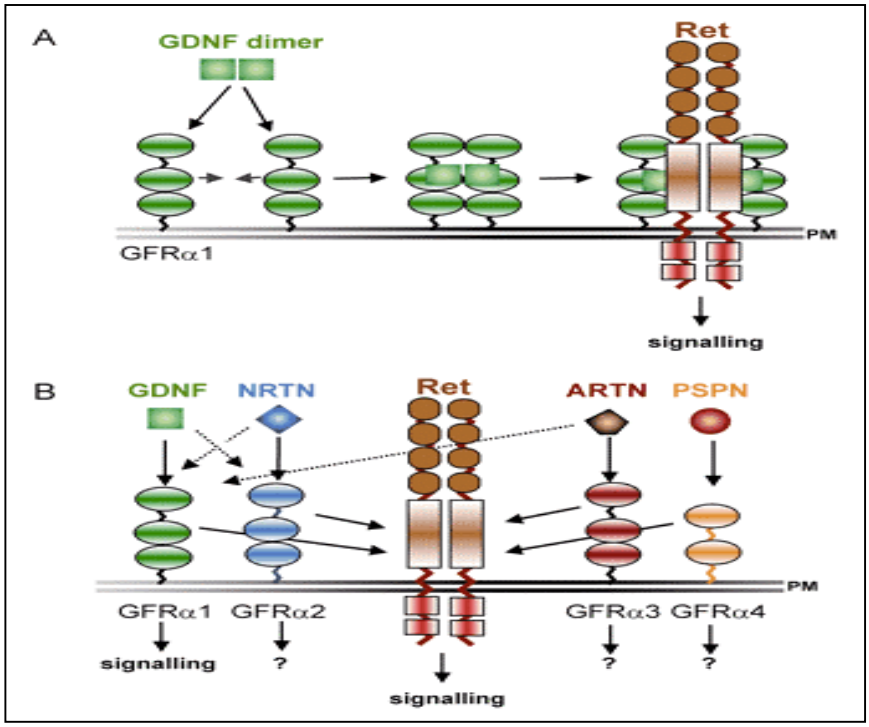


Fig 1.1 Signaling of the GDNF family members (Airaksinen and Saarma, 2002; Sariola and Saarma, 2003)

(A) A dimer of GDNF forms a complex with two molecules of GFRα1. This complex dimerizes two molecules of Ret. (B) Different GFLs activate Ret tyrosine kinase via different GFRα receptors. Solid arrows show the preferred ligand-receptor interactions, whereas dotted arrows show putative crosstalk (Airaksinen and Saarma, 2002; Sariola and Saarma, 2003).

four tyrosine residues (Tyr905, Tyr1015, Tyr1062 and Tyr1096) can be phosphorylated after Ret activation. One tyrosine residue can be the binding site for more than one docking protein; for example, many adaptors including Src-homologous and collagen-like protein (Shc), fibroblast growth factor receptor substrate 2 (FRS2), downstream of tyrosine kinase 4/5 (DOK4/5) and insulin receptor substrate 1/2 (IRS1/2) can bind to Tyr1062 in the carboxyterminal cytoplasmic tail of Ret (see Fig 1.2).

The mitogen-activated protein Kinase (MAPK) pathway is involved in the intracellular signaling of GDNF (see Fig 1.2) (Airaksinen and Saarma, 2002). The MAPK pathways transduces a large variety of external signals and is involved in diverse biological processes, including mRNA translation, cell proliferation and survival, and the nuclear genomic response to mitogens and cellular stresses (Roux and Blenis, 2004). Ras protein is a key component in MAPK pathways. Ras can bind to the adaptor protein for Ret (GFLs coreceptor) (see Fig 1.2);

The PI3-kinase enzymes are a group of ubiquitously expressed proteins that are involved in the PI3-kinase signaling pathway, which is involved in essential cellular functions such as survival, proliferation, migration and differentiation (Dancey, 2004). The adaptor protein for Ret (GFLs coreceptor) can recruit PI3K; therefore, the PI3K pathway can be involved in the intracellular signaling of GDNF (see Fig 1.2) (Airaksinen and Saarma, 2002).

The involvement of MAPK and PI3K pathways in the intracellular

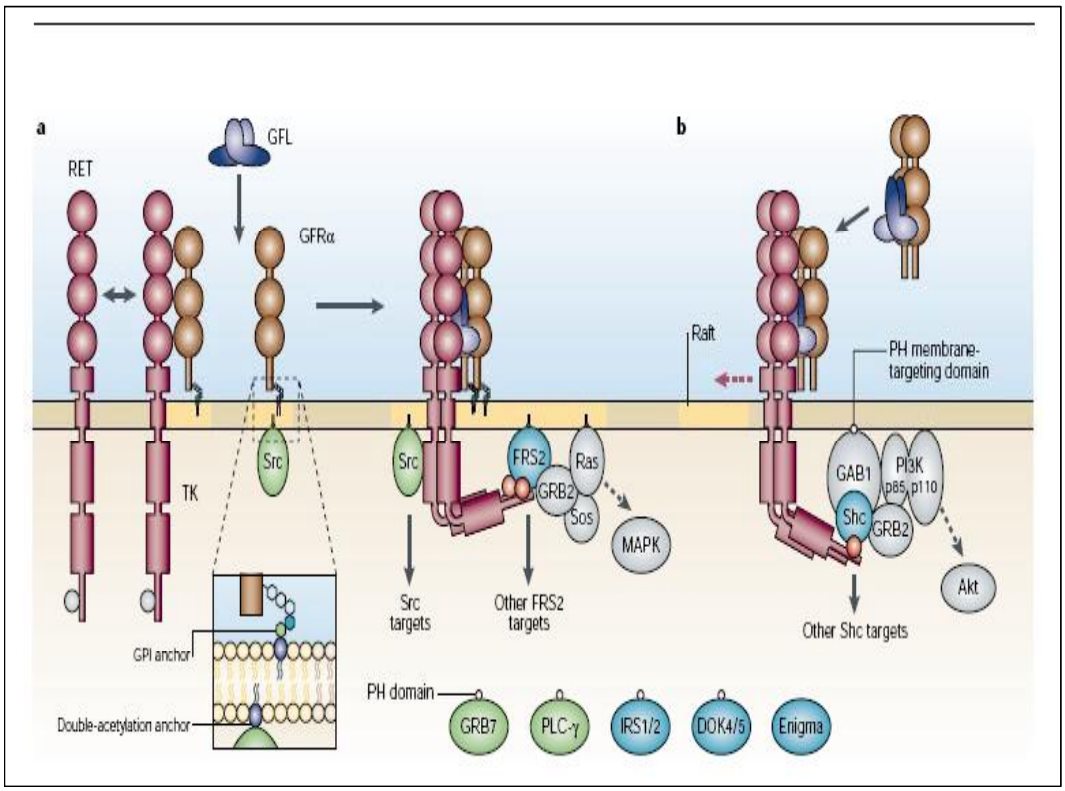


Fig 1.2 Intracellular signaling of GDNF (Airaksinen and Saarma, 2002)

The figure shows that when different adaptors bind to the tail of Ret, both PI3-kinase and MAPK pathways can be activated. The phosphorylated tyrosine residue (Tyr1062) in Ret can be bound by soluble adaptors such as Shc, FRS2, IRS1/2, DOK/4/5 and enigma.

Shc (Src-homologous and collagen-like protein), FRS2 (fibroblast growth factor receptor substrate 2), DOK4/5 (downstream of tyrosine kinase 4/5), GAB1, (GRB2-associated binding protein 1); GRB, (growth factor receptor-bound protein); MAPK, (mitogen-activated protein kinase); PI3K, (phosphatidylinositol 3-kinase; PLC, phospholipase C).

transduction indicates the wide spread function of GDNF might be related to these two pathways.

There is some evidence that some cells of neuronal origin which express GFRα-1, but not Ret, can be activated by GDNF, so it is possible that there is a signaling pathway independent of Ret for GDNF (Airaksinen et al., 1999).

1.8.3 Functions of GDNF and implication for Parkinson's disease

GDNF was found first as a growth factor for the embryonic dopaminergic neurons of the midbrain (Saarma, 2000); later it was found that it was also a trophic factor for spinal motor neurons and central noradrenergic neurons (Henderson et al., 1994; Arenas et al., 1995). GDNF and other GDNF family members not only target the CNS; some members can also affect the development of enteric, sympathetic and parasympathetic neurons (Sariola and Saarma, 2003).

One should highlight the potential use of GDNF to treat Parkinson's disease (PD). PD is caused by a progressive loss of nigral dopamine neurons and a concomitant decline of striatal dopamine function (Bjorklund et al., 2000), so blocking or slowing down the ongoing degenerative process should be the best method to treat this disease. Because of GDNF's protective effect on dopaminergic neurons, GDNF is a promising trial candidate for the treatment of PD. Intracerebral injections of recombinant GDNF have shown that GDNF can almost completely protect nigral dopamine neurons against 6-hydroxydopamine

(6-OHDA)- or MPTP-induced damage (Gash et al., 1998; Bjorklund et al., 2000).

GDNF works not only in the CNS, it can also affect the development of the kidney; for example, Ret-, GDNF-, and GFRα1-deficient mice do not develop kidneys (Enomoto et al., 1998; Manji et al., 2000). Studies have also found that GDNF is also involved in the differentiation of spermatogonial cells (Meng et al., 2000).

1.8.4 Regulation of the release of GDNF from C6 cells and astrocytes

1.8.4.1 Modulation of the release of GDNF by biological factors

Diverse biological factors including cytokines, neurotrophins, growth factors, neuropeptides, and pharmacological agents can modulate the GDNF level in C6 cells (Verity et al., 1998). For example, 24-h treatment with FGF-1, -2, or -9 increased the amount of GDNF secreted by C6 cells cultured in serum-free medium by five- to 10-fold compared to control. The proinflammatory cytokines including interleukin-1 beta, interleukin-6, tumor necrosis factor-alpha, and lipopolysaccharide can also increase GDNF release 1.5- to two-fold. By contrast, forskolin and dibutyryl cyclic AMP, which can be used to induce differentiation of C6 cells (Takanaga et al., 2004), decreased GDNF secretion (Verity et al., 1998). Thus it appears that differentiated C6 cells do not have an increased basal GDNF secretion as might be expected.

1.8.4.2 Modulation of the release of GDNF by antidepressants

As mentioned before, the upregulation BDNF may be a mechanism of action of antidepressants. Many studies suggest that GDNF may play a role in maintaining neuronal circuits in the mature CNS (Pochon et al. 1997; Trupp et al. 1997; Golden et al. 1998). Therefore it was speculated that antidepressants might have some effects on GDNF (Hisaoka et al., 2001).

Several antidepressants can increase GDNF mRNA levels and the secretion of GDNF in rat C6 glioblastoma cells. It was concluded that the regulation of GDNF release and synthesis by antidepressants was at the transcriptional level (Hisaoka et al., 2001).The antidepressants tested included amitriptyline, clomipramine, mianserin, fluoxetine and paroxetine at concentrations ranging from 1 to 25 μM, and the release of GDNF was dose-dependent. The secretion of GDNF was significant at 48 h treatment, but not at 24 h.

In the experiments of Hisaoka and colleagues, amitriptyline-induced GDNF release could be partially blocked by U0126, which is an inhibitor of mitogen-activated protein kinase (MAPK)-extracellular signal-related kinase (ERK) kinase (MEK), but could not be blocked by either H-89, a protein kinase A inhibitor, calphostin C, a protein kinase C inhibitor, or PD 169316, a p38 mitogen-activated protein kinase inhibitor. It was concluded that the GDNF release and synthesis was regulated by the MEK/MAPK signaling (Hisaoka et al., 2001).

It was also found that amitriptyline treatment increased GDNF mRNA

expression in rat astrocytes (Hisaoka et al., 2001). This finding suggests that GDNF modulation by antidepressants may also apply to primary glial cells.

1.8.4.3 Modulation of the release of GDNF by monoamines

Antidepressants can increase the available concentration of monoamines in the synapse, so it is easy to speculate that the effect of antidepressants on the release of GDNF is mediated by increased available concentration of monoamine transmitters. It was found that serotonin treatment, but neither dopamine nor noradrenaline, can upregulate the synthesis and release of GNDF in a dose- and time-dependent manner in C6 cells (Hisaoka et al., 2004). Significant release of GDNF occurred after 48 h treatment with 100 μM of the monoamine serotonin. This serotonin-induced release of GDNF was partially blocked by pretreatment with ketanserin or cyproheptadine, both of which are 5-HT2A receptor antagonists. This suggests that the 5-HT2A receptor may be involved in the regulation of GDNF release in C6 cells. However, since the effect of serotonin was also partially blocked by U0126 (Hisaoka et al., 2004), this suggests that the MEK/MAPK signaling pathway is also involved in the regulation of GDNF release. Several papers demonstrated that the MEK/MAPK signaling pathway can be activated via 5-HT2A receptor (Watts, 1998; Banes et al., 1999; Johnson-Farley et al., 2005); therefore it is possible that the synthesis and release of GDNF caused by serotonin is mediated by the 5-HT2A receptor and MAPK signaling.

1.8.4.4 Modulation of the release of GDNF by MAOIs

Other researchers studied the regulation of GDNF by monoamine oxidase (MAO) inhibitors in cultured astrocytes. It was found that the MAO-B inhibitors, selegiline and desmethylselegiline, can induce NGF, BDNF, and GDNF gene expression and secretion of these trophic factors into the culture medium (Mizuta et al., 2000). The concentrations of selegiline used, however, were much higher than the concentration (0.2mM) required for the complete inhibition of MAO. Selegiline at 0.2 mM completely inhibited the MAO activity, but had no effect on the content of neurotrophic factors; therefore, it was concluded that stimulation of neurotrophic factor release by selegiline was independent of MAO-B inhibition (Mizuta et al., 2000).

1.8.4.5 Modulation of the release of GDNF by dopamine receptor agonists

Bromocriptine, a dopamine D2 receptor agonist, elevated NGF levels in the culture medium of mouse astrocytes, and significantly decreased GDNF and BDNF levels at 24 h. Both pergolide, a D1/D2 receptor agonist, and cabergoline, a D2/weak D1 receptor agonist, rapidly elevated NGF and GDNF levels at 4-6 h. SKF-38393, a D1 receptor agonist, elevated NGF and GDNF levels at 24 h (Ohta et al., 2003). The increase of GDNF and NGF were probably due to transcription, because a corresponding elevation of mRNA was also found. These findings suggest that stimulation of dopamine D1 receptors can increase the synthesis of GDNF and NGF, while stimulation of dopamine D2 receptors

may inhibit NGF and GDNF synthesis (Ohta et al., 2003).

1.8.4.6 Modulation of the release of GDNF by antipsychotic drugs

In the experiments of Hisaoka and colleagues, the antipsychotic drug haloperidol (1 μM) was also used. It was found that haloperidol had no effect on the release of GDNF from C6 cells (Hisaoka et al., 2001).

Previous work from our laboratory, however, found that the atypical antipsychotic drugs, quetiapine and clozapine, and the typical antipsychotic drug, haloperidol, increased the secretion of GDNF from C6 cell after 48 h treatment (Shao et al., 2006). Compared to results of Hisaoka and colleagues, Shao and colleagues found that haloperidol (greater than 10 μM) could increase the GDNF release from C6 cells. So the lack of haloperidol-induced GDNF release in the experiments of Hisaoka and colleagues was due to the lower concentration of haloperidol that they tested (Shao et al., 2006; Hisaoka et al., 2001).

Shao and colleagues did not find any difference between atypical and typical antipsychotics in the regulation of GDNF from C6 cells (Shao et al., 2006). This suggests that modulation of GDNF release is not specific for atypical antipsychotics.

Shao and colleagues also measured the intracellular level of GDNF in C6 cells after antipsychotic treatment. No difference was found in the intracellular level of GDNF between the treatment groups and the control group. Shao and colleagues concluded that the increased release of GDNF from C6 cells after

treatment was not caused by non-specific leakage from damaged cells, but by increased synthesis (Shao et al., 2006).

As with Hisaoka and colleagues, Shao and colleagues did not find any change in the number of C6 cells after antipsychotic treatment (Hisaoka et al., 2001; Shao et al., 2006), although GDNF can have a proliferative effect on C6 cells (Suter-Crazzolara et al., 1996).

Shao and colleagues concluded that GDNF release from C6 cells was stimulated by quetiapine (5 to 25 μM), clozapine (5 to 25 μM) and haloperidol (10 to 25 μM). The increase in the release of GDNF caused by antipsychotic drugs may be a possible mechanism of antipsychotic drugs in the treatment of neurodegenerative diseases (Shao et al., 2006).

From all of the above studies, it can be concluded that besides the biological factors such as cytokines, neurotrophins, the growth factors and neuropeptides that can modulate the synthesis and secretion of GDNF, there are at least two neurotransmitters systems, dopamine and serotonin, both of which can be involved in the regulation of synthesis and secretion of GDNF. Antidepressants, amongst many other actions, can increase the available concentration of serotonin in synapse, and similarly, antipsychotic drugs, amongst many pharmacological actions, are antagonists of D2 receptors. As such, both antidepressants and antipsychotics may be involved in the regulation of GDNF. The role of antidepressants in the regulation of GDNF was demonstrated in the study of Hisaoka and colleagues (Hisaoka et al., 2001),

while experiments from our laboratory (Shao et al., 2006) indicated that antipsychotics can modulate the expression and secretion of GDNF in C6 cells.

1.9 Hypotheses

According to the literature, a decrease in glial cell number or a hypofunction of glial cells in depression exists, and this dysfunction may induce an aberrant environment for neurons, i.e., a lower that normal level of neurotrophic factors. Both antidepressants and antipsychotics may target glial cells. **The hypothesis tested in this thesis states that antidepressants and antipsychotics may affect the proliferation, differentiation and function of glial cells**. For example, antidepressants may increase the level of neurotrophic factors such as GDNF released from glial cells, especially astrocytes. The increased GDNF may improve the function of both glial cells and neurons. Atypical antipsychotics may have similar effects as antidepressants as they also increase the release of GDNF from C6 cells (Shao et al., 2006) and have shown some capacities to treat depression.

C6 cells will be used as a model for glial cells. Both an antidepressant (fluoxetine) and an antipsychotic drug (quetiapine) will be used to study their effects on the proliferation, differentiation, and release of GDNF release from C6 cells. Combinations of these drugs will be used to study potential synergistic effect on the release of GDNF from C6 cells.

1.9.1 Experimental designs

This research project had three main goals:

1. To investigate the effects of quetiapine and fluoxetine on the proliferation and differentiation of C6 cells.
2. To investigate the effects of quetiapine and fluoxetine on the release of GDNF.
3. To investigate whether there is synergistic interaction between quetiapine and fluoxetine on the release of GDNF.

Five experiments were designed to fulfill these goals.

Experiment 1 - Cell number (MTT assay)

To investigate the effects of quetiapine or fluoxetine treatment on C6 cell number. Cell number was studied under serum present and serum starvation conditions. Both conditions were chosen, because serum starvation was the condition used for study about GDNF release, while serum present condition was used for a comparison.

Experiment 2 – Proliferation (BrdU ELISA))

To investigate the effects of quetiapine and fluoxetine on the proliferation of C6 cells under serum present and serum starvation conditions.

Experiment 3 – Mortality (Live-Dead cell kit)

To investigate the effects of quetiapine and fluoxetine on C6 cell mortality.

Experiment 4 – Morphology (GFAP staining)

To investigate the effects of quetiapine and fluoxetine treatment on the morphology of C6 cells.

Experiment 5 – GDNF release (ELISA)

To investigate the effects of quetiapine and fluoxetine treatment, either separately or combined, on GDNF release.

2. Methods

2.1 Cell culture

C6 cells were obtained from the American Type Culture Collection (ATCC) (37 passages when ordered). The cells used were between passages 37 and 47. C6 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified environment. For the serum starvation condition, no FBS was added and the OptiMEM (Gibco) was supplemented with 0.5% bovine serum albumin (BSA).

2.2 MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] conversion assay is based on the ability of a dehydrogenase enzyme in mitochondria in viable cells to cleave the tetrazolium rings of the pale yellow MTT into a dark blue formazan product, which is largely impermeable to cell membranes, thus resulting in its accumulation within viable cells. Addition of a detergent to cultures causes the liberation of the crystals. The level of the formazan product created can reflect the number of viable cells. The color can then be quantified with a microplate reader. The detailed protocol was:

1. Prepare the cells needed for MTT assay (after treatment), remove the media from cultured cells.
2. Prepare the MTT working solution.

3. Add MTT working solution into wells being assayed (100 μl/well), incubate at 37 °C for 2 h.
4. At the end of the incubation period, remove the medium.
5. The converted dye is solubized with 100 μl DMSO/well.
6. Absorbance of the converted dye is measured at a wavelength of 570 nm.

MTT stock solution: 5 mg/ml MTT (Promega) in PBS

MTT working solution: 1:10 dilution of the 5 mg/ml stock with culture medium.

2.3 BrdU enzyme linked immunosorbent assay (ELISA)

To measure the proliferation of cells, a commercially available kit (Cell Proliferation ELISA, BrdU Colorimetric kit, Roche Diagnostics) was used to measure the BrdU incorporation during DNA synthesis. It is a non-radioactive alternative to the [^{3}H]-thymidine incorporation assay. The detailed protocol was:

1. Cells were cultured under desired condition (or under different treatment) in 96-well plates.
2. At the appropriate time, 10 μl/well of BrdU labeling solution was added into each well, and the cells were incubated for 2 h at 37°C.
3. Remove labeling medium.
4. Add 200 μl/well FixDenat to cells, and incubate for 30 min at 15-25°C.
5. Remove FixDenat solution thoroughly.

6. Add 100 μl/well anti-BrdU-POD working solution and incubate for 90 min at room temperature.
7. Remove antibody conjugate by flicking off and wash wells three times with 300μl/well washing solution.
8. Remove washing solution by tapping; add 100 μl/well substrate solution; incubate for 25 min at room temperature.
9. Measure the absorbance of the samples in a microplate reader at 370 nm.

2.3.1 Solutions for BrdU ELISA

BrdU labeling solution: dilute BrdU labeling reagent 1:100 with sterile culture medium.

Anti-BrdU-POD stock solution: dissolve anti-BrdU-POD in 1.1 ml double distilled water for 10 min and mix thoroughly.

Anti-BrdU-POD working solution: dilute anti-BrdU-POD stock solution 1:100 with antibody dilution solution.

Washing solution: dilute washing buffer concentrate 1:10 with double distilled water.

FixDenat: solution provided in the kit.

2.4 Live-Dead cell kit for mortality

The viability/cytotoxicity assay kit for Live & Dead cells (Biotium 30002) is a two-color fluorescence staining method; two probes that measure intracellular

esterase activity and plasma membrane integrity can distinguish between live and dead cells.

Live cells are permeable to calcein AM, which can be converted to the intensely fluorescent calcein by intracellular esterase activity, producing a green fluorescence. Cells with damaged membranes are permeable to EthD-III, which binds to nucleic acids. A red fluorescence in dead cells is produced.

The detailed protocol was:

1. Remove the Calcein AM and Eth-III reagent stock solutions from the freezer and allow them warm to room temperature for 30 min.
2. Add 20μL of the supplied 2 mM EthD-III stock solution to 10 mL of PBS, vortexing to through mixing to get 4 μM EthD-III solution.
3. Combine the reagents by transferring 5 μL of the 4 mM calcein AM stock solution to the 10 mL EthD-III solution.
4. Wash the 8-well chamber with 500 μl PBS and aspirate out the supernatant.
5. Add 200 μl of Calcein AM/EthD-III standard staining solution.
6. Incubate the cells for 30 min at room temperature.
7. Aspirate out Calcein AM/EthD-III standard staining solution and mount a coverslip. Seal the coverslip with mounting media.
8. View the labeled cells under fluorescence microscope.

2.5 GFAP staining of C6 cells

1. Fix cells with 4% paraformaldehyde (PF) for 30 min.
2. Wash 3 times with PBS for 10 min each.
3. Block the sample with 1% BSA for 30 min.
4. Incubate with anti-GFAP (1:1000) antibody at 4°C overnight.
5. Wash with PBS 3 times.
6. Incubate with anti-mouse antibody conjugated with Cy^3.
7. Wash with PBS 3 times.
8. Mount the coverslip with mounting media.
9. Observe with a fluorescence microscope and take photographs.
10. Measure the mean fluorescent intensity of GFAP staining with Image Plus software.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA) for GDNF

The GDNF protein concentration in the supernatants of cell cultures were detected using a GDNF ELISA kit according to the manufacture's instructions (Promega, Madison , WI, USA). The detailed protocol was:

1. 96-well ELISA plates were coated with anti-GDNF monoclonal antibody diluted in ELISA coating buffer (1:1000 dilution, 100 μl/well) and incubated overnight at 4°C.
2. After emptying the coating buffer from each well, plates were blocked with 1× Block and Sample Buffer (Promega; 200 μl/well) for 1 h at

room temperature.

3. GDNF standards from 0 pg/mL to 1000 pg/mL and samples 1:1 diluted with1× Block and Sample buffer were added in duplicate to plates (100 μl/well), and shaken at 500 rpm for 6 h.
4. After plates were washed five times with TBST buffer, 100 μl/well anti human polyclonal antibody (1:500 dilution with 1× Block and Sample Buffer) were added to each well, and incubated overnight at 4°C.
5. After plates had been washed five times with TBST, 100 μl/well horseradish peroxidase-conjugated anti-chicken antibody (1:250 dilution with 1× Block and Sample Buffer) were added to each well and incubated at room temperature, with shaking at 500 rpm for 2 h.
6. After the plates had been washed five times with TBST, 100 μl/well TMB one solution (Promega) was added to each well, and incubated for 15 min at room temperature in the dark.
7. The reaction was stopped by adding 100 μl 1 N hydrochloric acid to each well, and the absorbance at 450 nm (A450) was recorded on the microplate reader (Molecular Devices Spectra Max Plus 384). The linear relationship between A450 and GDNF concentration was from 15 to 1000 pg/mL. The sample GDNF concentration was determined from the GDNF standard curve.

2.6.1 Solutions for the GDNF ELISA assay

Carbonate coating buffer (pH 8.2):

0.025 M sodium bicarbonate (Sigma S-5761, FW 84.01)

0.025 M sodium carbonate (Sigma S-2127, FW 106.0)

TBST wash buffer:

20 mM Tris Hydrochloride (pH 7.6) (Sigma T-8404, FW 157.60)

150 mM NaCl (BDH, FW 58.44)

0.05 %(v/v) Tween 20

Dilution of GDNF standard:

Standard 2μg/ml	10μl
1× block and sample buffer	390 μl 1:40 dilutions
1:40 dilution standard	10 μl
1× block and sample buffer	490 μl1: 2000 dilution (1000 pg/ml)

Serial dilution of GDNF standard:

Concentration (pg/ml)	1000	500	250	125	62.5	31.25	15.6
Standard (μl)	230	230	230	230	230	230	230
Buffer (μl)	230	230	230	230	230	230	230

230 μl stock solution of GDNF (1000 pg/ml) standard and 230 μl 1×block and sample buffer were mixed thoroughly to get GDNF standard of 500 pg/ml.

Repeat the above step 6 to make the next dilution, using the first dilution (the 500 pg/ml solution in this case) as new stock solution.

Repeat 6 times until 15.6 pg/ml GDNF solution was made.

2.7 Protocols for individual experiments

2.7.1 The effects of quetiapine and fluoxetine on C6 cell number

1×10^4 C6 cells/well in DMEM (100 μl/well) with 10% FBS supplement were seeded into the inner 40 wells of a 96-well plate (peripheral wells were not used for experiment, but filled with medium). After 24 h at 37 °C in a 5% CO_2 environment, the medium was changed to fresh DMEM or to OptiMEM with different concentrations of quetiapine (1, 6.25, 12.5 and 25 μM) or fluoxetine (1, 6.25, 12.5 and 25 μM); 6 wells for each concentration. This was repeated four times. C6 cells were grown for 12, 24, 36 and 48 h in 5% CO_2 environment after treatment. The MTT assay was used to measure the number of viable cells.

Drugs diluted in OptiMEM (with 0.5% BSA supplement) after DMEM removal (with 10% FBS supplement) was the serum starvation condition in these experiments, while drugs diluted in fresh DMEM (with 10% FBS supplement) after DMEM removal was the serum present condition in my experiments.

2.7.2 The effect of fluoxetine and quetiapine on C6 cell proliferation

1×10^4 C6 cells/well in DMEM (100 μl/well) with 10% FBS supplement were seeded into the inner 40 wells of 96-well plates. After 24 h incubation at 37 °C in a 5% CO_2 atmosphere, the medium was changed to DMEM or OptiMEM with quetiapine or fluoxetine (1, 6.25, 12.5, 25 μM), 6 wells for each

concentration. This was repeated four times. C6 cells were grown for 24 h. After drug treatment, BrdU ELISA was used to measure the proliferation of C6 cells.

2.7.3 The mortality of C6 cells after quetiapine and fluoxetine treatment

2×10^4 C6 cells/chamber in DMEM (600 μl/chamber) with 10% FBS supplement were seeded into 8-well chamber slides. After 24 h incubation at 37°C in a 5% CO_2 atmosphere, the medium was changed to OptiMEM (with 0.5% FBS supplement) with 25 μM quetiapine or 12.5 μM fluoxetine. C6 cells were grown for 24 or 48 h in a 5% CO_2 atmosphere. After treatment, the numbers of live and dead C6 cells were determined with the Live & Dead cell assay kit. Three photographs were taken of each well under a fluorescence microscope at 100 times magnification. Live and dead cells were counted, and the percentage of dead cells was calculated.

2.7.4 The morphology and GFAP staining of C6 cells after fluoxetine and quetiapine treatment

5×10^4 C6 cells/well in DMEM (1 ml/well) with 10% FBS supplement were seeded onto a coverslip in each well of a 24-well plate. After 24 h incubation, the medium was changed to OptiMEM (with 0.5% BSA supplement) with 25 μM quetiapine or 25 μM fluoxetine. The C6 cells were grown for 24 or 48 h in a 5% CO_2 atmosphere. After treatment, GFAP staining was performed. Three

photographs were taken for each well of C6 cells after GFAP staining under a fluorescence microscope at 200× magnification. The mean intensity of GFAP staining was calculated with Image Plus software.

2.7.5 The effects of fluoxetine and quetiapine on the release of GDNF

3×10^5 C6 cells/well in DMEM (1 ml/well) with 10% FBS supplement were seeded into 6-well plates. After 24 h incubation at 37°C in a 5% CO_2 atmosphere, the medium was changed to OptiMEM (with 0.5% BSA supplement) with different concentrations of quetiapine (1, 6.25, 12.5 and 25 μM) or fluoxetine (1, 6.25, 12.5 and 25 μM); three wells were used for each concentration. C6 cells were grown for 48 h in 5% CO_2 atmosphere, after drug treatment. The medium was collected and after centrifugation at 1200 rpm at 4°C for 10 min, the supernatant was collected, and stored at -20°C. The supernatant was used for the GDNF ELISA assay.

For the time-course of the effects of fluoxetine (12.5 μM) or quetiapine (25 μM) on the GDNF release, the C6 cells were incubated at 37°C for 12, 24 and 48 h in a 5% CO_2 environment.

2.8 Data analysis

SPSS 13.0 for Windows was used to analyze the data for one-way ANOVA and two-way ANOVA. Post-hoc Tukey HSD test was used to compare the difference between groups.

3. Results

3.1. The effects of quetiapine and fluoxetine on MTT conversion

3.1.1 Experimental condition: serum present

In the serum present condition after 24 h culturing, quetiapine (1, 6.25, 12.5, 25 μM) and low concentrations of fluoxetine (1, 6.25, 12.5 μM) had no significant effect on MTT conversion, which can be used as an indication of the number of C6 cells. By contrast, 25 μM fluoxetine significantly decreased the number of C6 cells to 78±4% of control ($p<0.01$) (see Fig 3.1).

In the serum present condition after 48 h culturing, low concentrations of fluoxetine and low concentrations of quetiapine had no significant effect on the number of C6 cells (see Fig 3.2). However, 25 μM fluoxetine significantly decreased the number of C6 cells to 53±6% of control ($p<0.01$). Similarly, 25 μM quetiapine significantly deceased the number of C6 cells to 83±2% of control ($p<0.05$) (see Fig 3.2). Thus, the decreased number of C6 cells caused by quetiapine and by fluoxetine occurred only at high concentrations.

The effects of fluoxetine and quetiapine on the number of C6 cells were time-dependent (see Fig 3.3). Fluoxetine (25 μM) decreased the number of C6 cells at an earlier time point (24 h) than did quetiapine, and although both drugs affected cell number by 48h, the effect of fluoxetine was still greater.

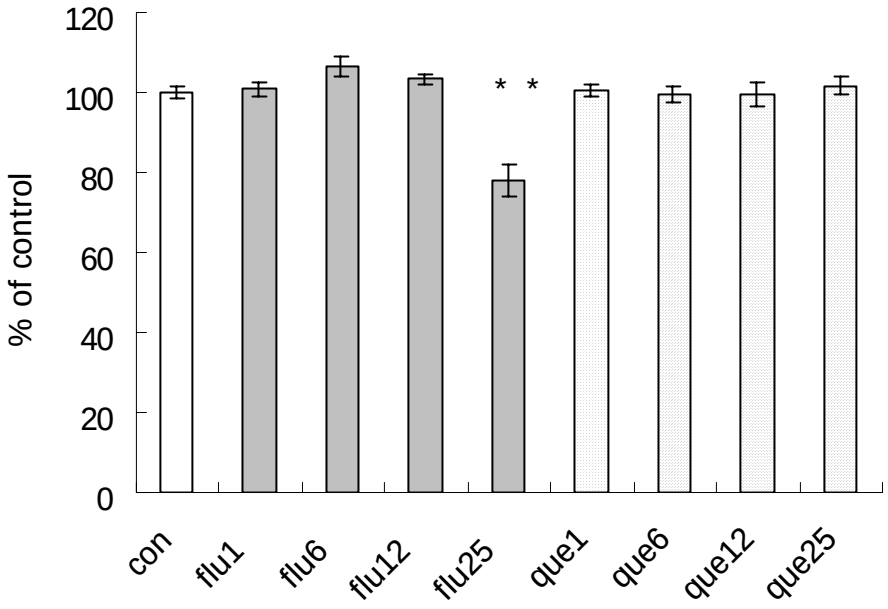


Fig 3.1 The MTT conversion after 24 h fluoxetine or quetiapine treatment in the serum present condition. Values shown are percentage of control MTT conversion [mean±SEM (n=4)]. ** $p<0.01$ compared to control.

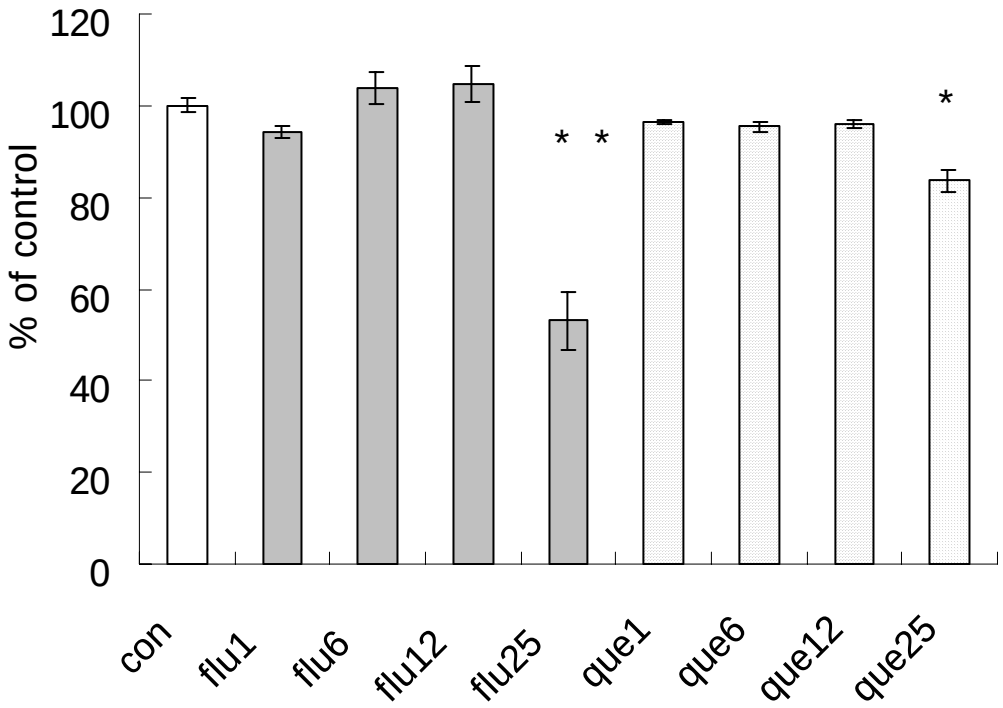


Fig 3.2 The MTT conversion after 48 h fluoxetine or quetiapine treatment in the serum present condition. Values shown are percentage of control MTT conversion [mean±SEM (n=4)]. ** $p<0.01$ compared to control.

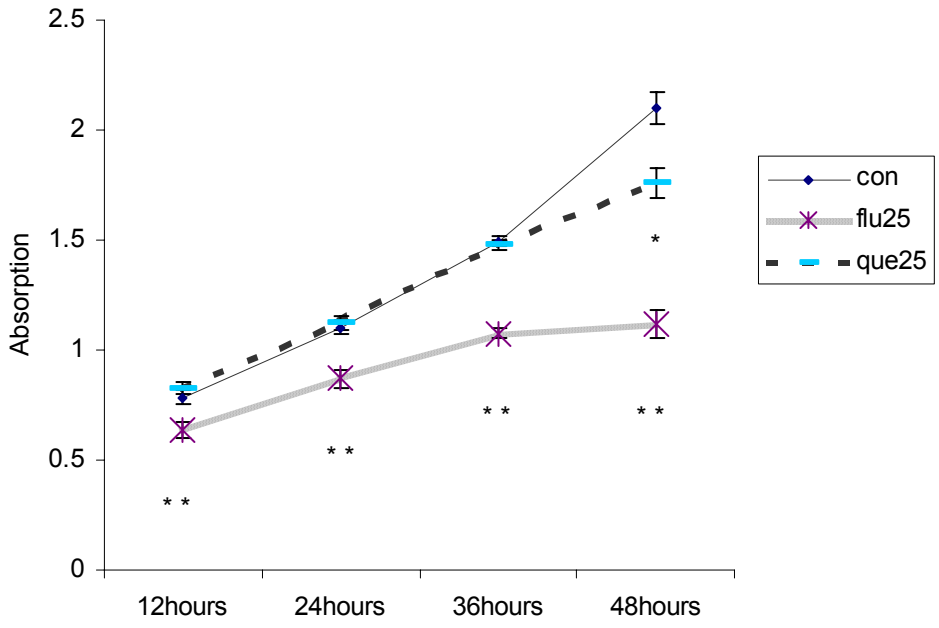


Fig 3.3 The effects of time in culture (serum present) on the MTT conversion. Values shown are absorption (OD at 570 nm) [mean±SEM (n=4)]. ** $p<0.01$ * $p < 0.05$ compared to control.

3.1.2 Experimental condition: serum starvation

After 24 h in the serum starvation condition, the lowest concentration of fluoxetine (1 μM) had no significant effect on the MTT conversion (see Fig 3.4). However, higher concentrations of fluoxetine (6.25, 12.5, 25 μM) significantly increased the MTT conversion after 24 h culturing ($p<0.01$). A concentration of 12.5 μM fluoxetine induced the biggest increase in the MTT conversion (142±4% of control). Quetiapine had no significant effect on the MTT conversion after 24 h culturing at any of the concentrations tested (1, 6, 12.5, 25 μM) (see Fig 3.4).

At the 48 h time period in the serum starvation condition, 12.5 μM fluoxetine increased the MTT conversion to 116±6% of control ($p<0.05$) (see Fig 3.5). 1, 6.25 and 25 μM fluoxetine had no significant effect on the MTT conversion. Low concentrations of quetiapine (1, 6, 12.5 μM) had no significant effect on the MTT conversion, while 25 μM quetiapine decreased the MTT conversion to 87±4% of control ($p<0.05$) (see Fig 3.5).

The time dependency of the effects of quetiapine and fluoxetine on the MTT conversion in the serum starvation condition are shown in Fig 3.6. 25 μM quetiapine decreased the MTT conversion only after 48 h treatment. By contrast, 12.5 μM fluoxetine increased the MTT conversion significantly at 12,24, 36 and 48 h time periods (see Fig 3.6).

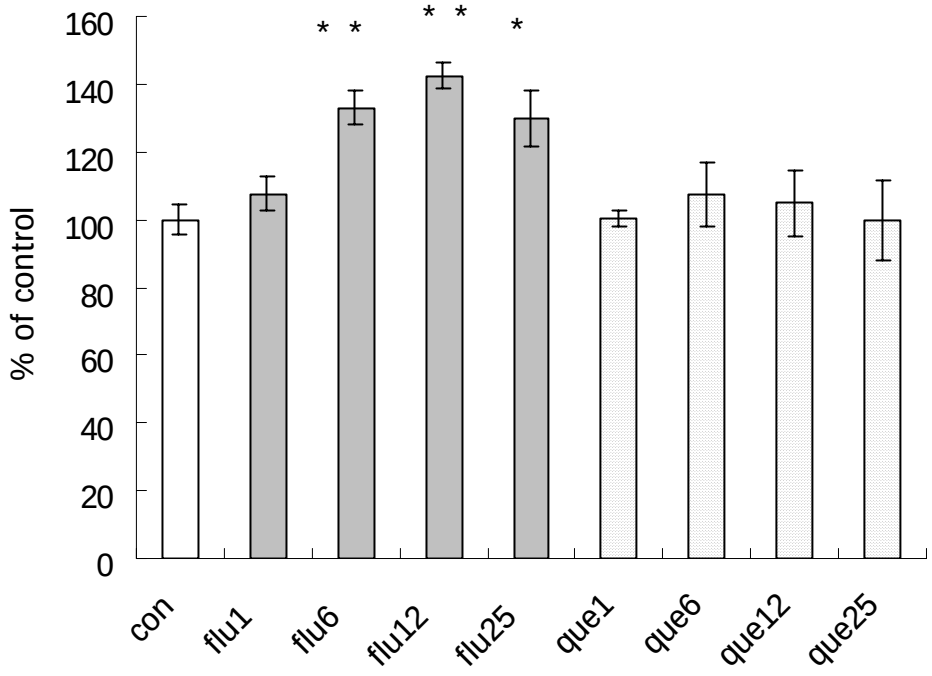


Fig 3.4 The MTT conversion after 24 h fluoxetine (flu) or quetiapine (que) treatment in the serum starvation condition. Values shown are percentage of control MTT conversion [mean±SEM (n=4)]. * $p<0.05$,** $p < 0.01$ compared to control.

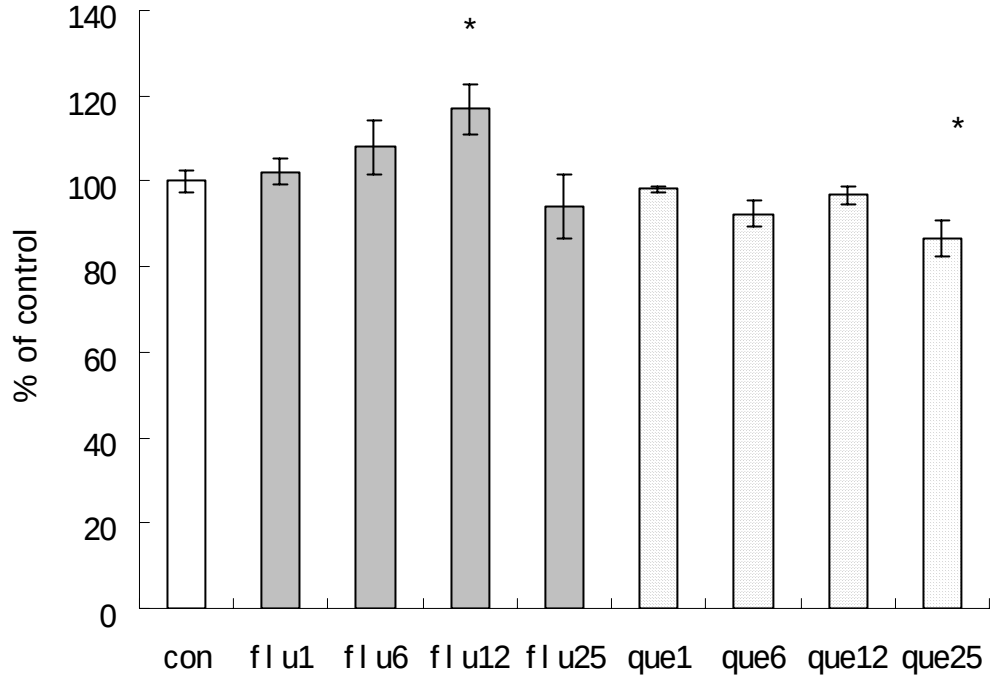


Fig 3.5 The MTT conversion after 48 h fluoxetine (flu) or quetiapine (que) treatment in the serum starvation condition. Values shown are percentage of control MTT conversion [mean±SEM (n=4)]. * $p<0.05$ compared to control.

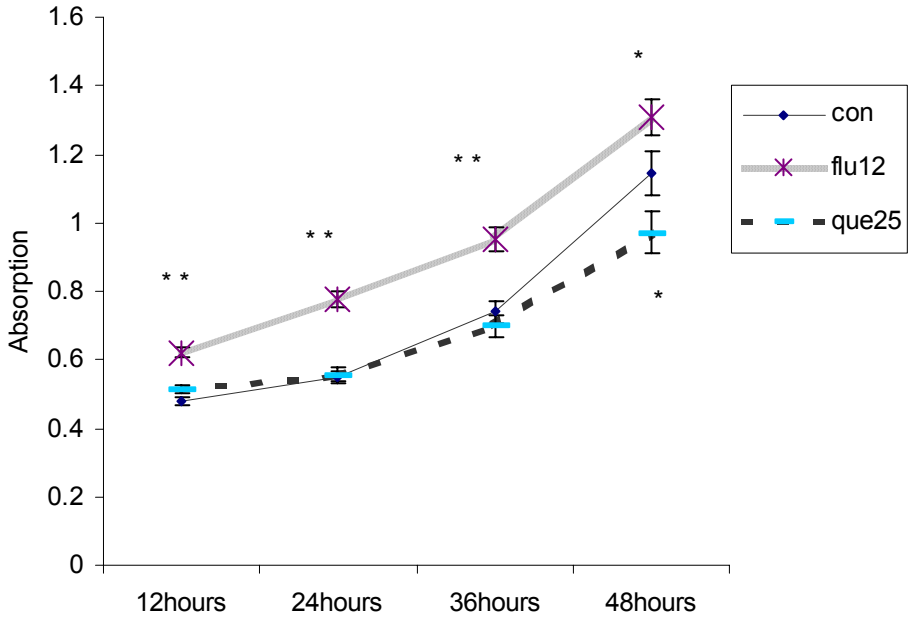


Fig 3.6 The effects of time in culture (serum starvation) on the MTT conversion. Values shown are absorption (OD at 570 nm) [mean ± SEM (n=4)]. ** $p<0.01$ * $p<0.05$ compared to control.

3.2 The effect of fluoxetine on C6 cell proliferation (BrdU incorporation)

3.2.1Experimental condition: serum present

In the serum present condition, 25 μM fluoxetine and 25 μM quetiapine decreased the proliferation of C6 cells to 77±1% of control and 79±3% of control, respectively ($p<0.01$) (see panel B Fig 3.8).

3.2.2 Experimental condition: serum starvation

In the serum starvation condition after 24 h culturing, fluoxetine inhibited the proliferation of C6 cells in a dose-dependent manner (see Fig 3.7. and Table 3.1), with 12.5 and 25 μM fluoxetine having significant effects on the proliferation of C6 cells. [89±2% of control and 55±1% of control, respectively ($p<0.01$)] (see Fig 3.7).

25 μM quetiapine decreased the proliferation of C6 cells to 86±3% of control at 24 h ($p<0.01$) (see panel A Fig 3.8).

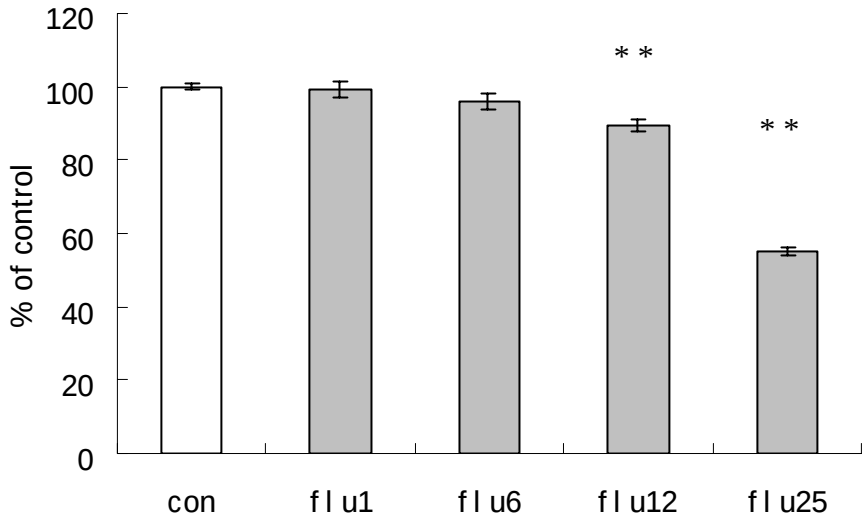
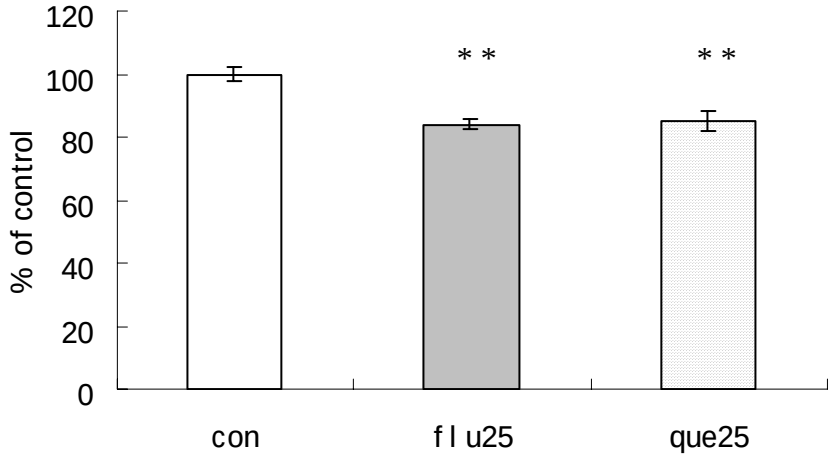


Fig 3.7 The effect of different concentrations of fluoxetine (flu concentrations in μM) on proliferation (BrdU incorporation) of C6 cells in the serum starvation condition after 24 h treatment. Values shown are mean±SEM (n=12). ** $P < 0.01$ compared to control.

A



B

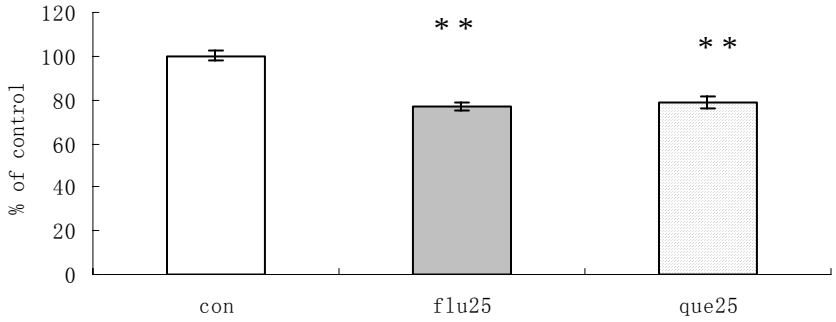


Fig 3.8 The effects of fluoxetine (flu concentration in μM) and quetiapine (que concentrations in μM) on the proliferation (BrdU incorporation) of C6 cell in the serum present (B) and serum starvation conditions (A) after 24 h treatment. Values shown are mean±SEM (n=12). * *p<0.01 compared to control.

3.3 The effects of fluoxetine and quetiapine on C6 cell death

Using the Live/ Dead assay kit, it was found that there were fewer dead C6 cells after 12.5 μM fluoxetine treatment (24h, serum starvation) (see Fig 3.9). For example, in the photograph shown, there are eight dead cells in the control, and four and six dead cells in the fluoxetine and quetiapine-treated samples. By calculating the percentage of dead cells from all photographs (three photographs were take for each well), it was found that 25 μM quetiapine did not affect the mortality of C6 cells. The percentages of dead cells in the control and the 25 μM quetiapine groups were 4.9±0.5% and 3.5±0.4%, respectively. 12.5 μM fluoxetine, however, decreased the mortality of C6 cells to 1.6 ±0.4% ($p<0.01$) (Fig 3.11 A)

After 48 h culturing, it was found that there were fewer dead cells following 12.5 μM fluoxetine treatment (Fig 3.10). For example, in the photograph shown, there are 14 dead cells in the control, and 5 and 10 dead cells in the fluoxetine and quetiapine-treated samples. By calculating the percentage of dead cells from all photographs, it was found that 25 μM quetiapine did not affect the mortality of C6 cells. The percentages of dead cells in the control and the 25 μM quetiapine groups were 6.0±0.9 % and 6.4±1.1%, respectively. However, fluoxetine (12.5 μM) decreased the mortality of C6 cells to 1.9±0.2% ($p<0.01$ compared to control) (see Fig 3.11 B).

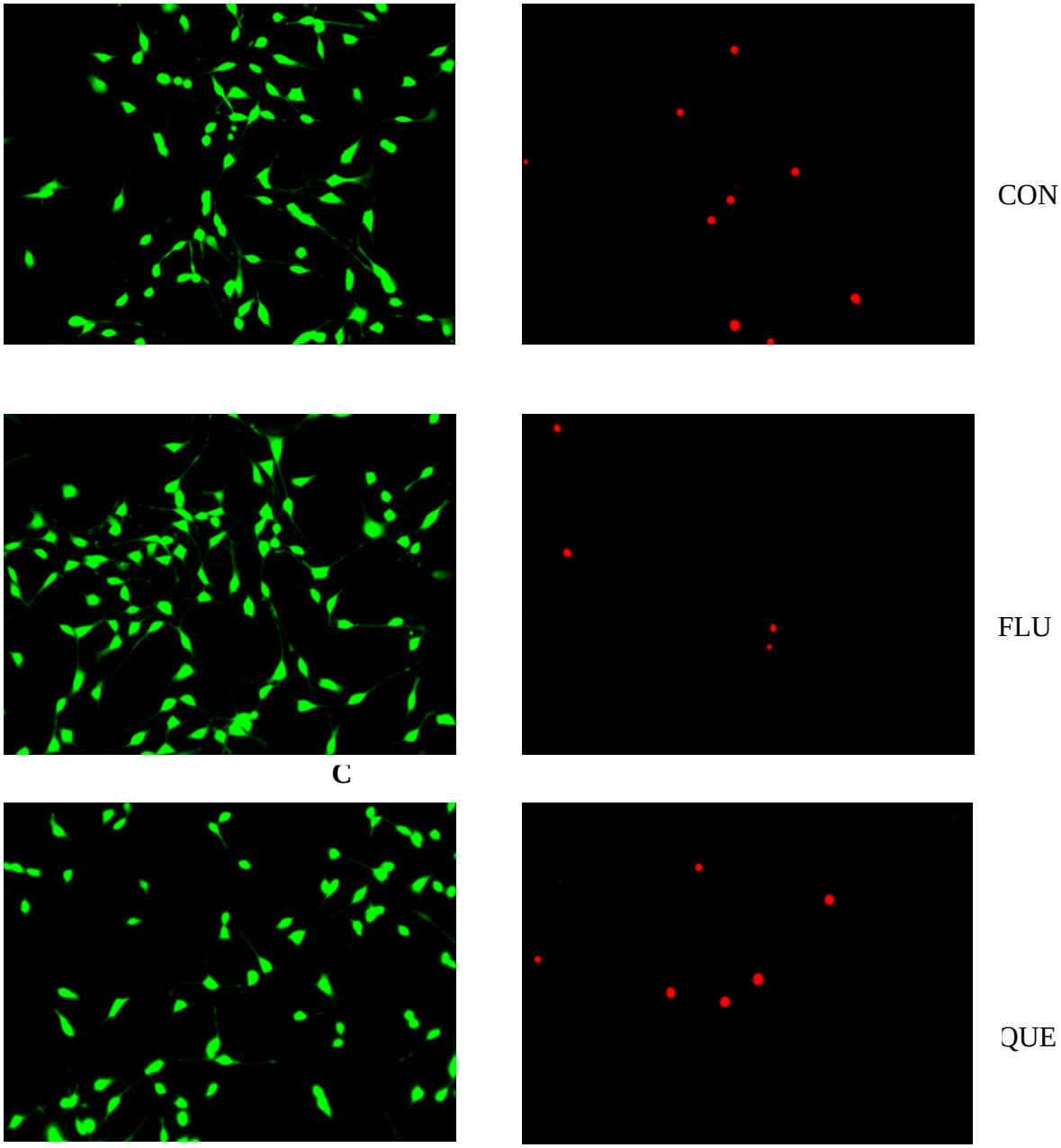


Fig 3.9 C6 cells stained by the Live - Dead Kit after 24 h treatment with quetiapine (25 μM) or fluoxetine (12.5 μM) under the serum starvation condition.

Magnification: 100×

Green color (left) :live cells Red color (right): dead cells

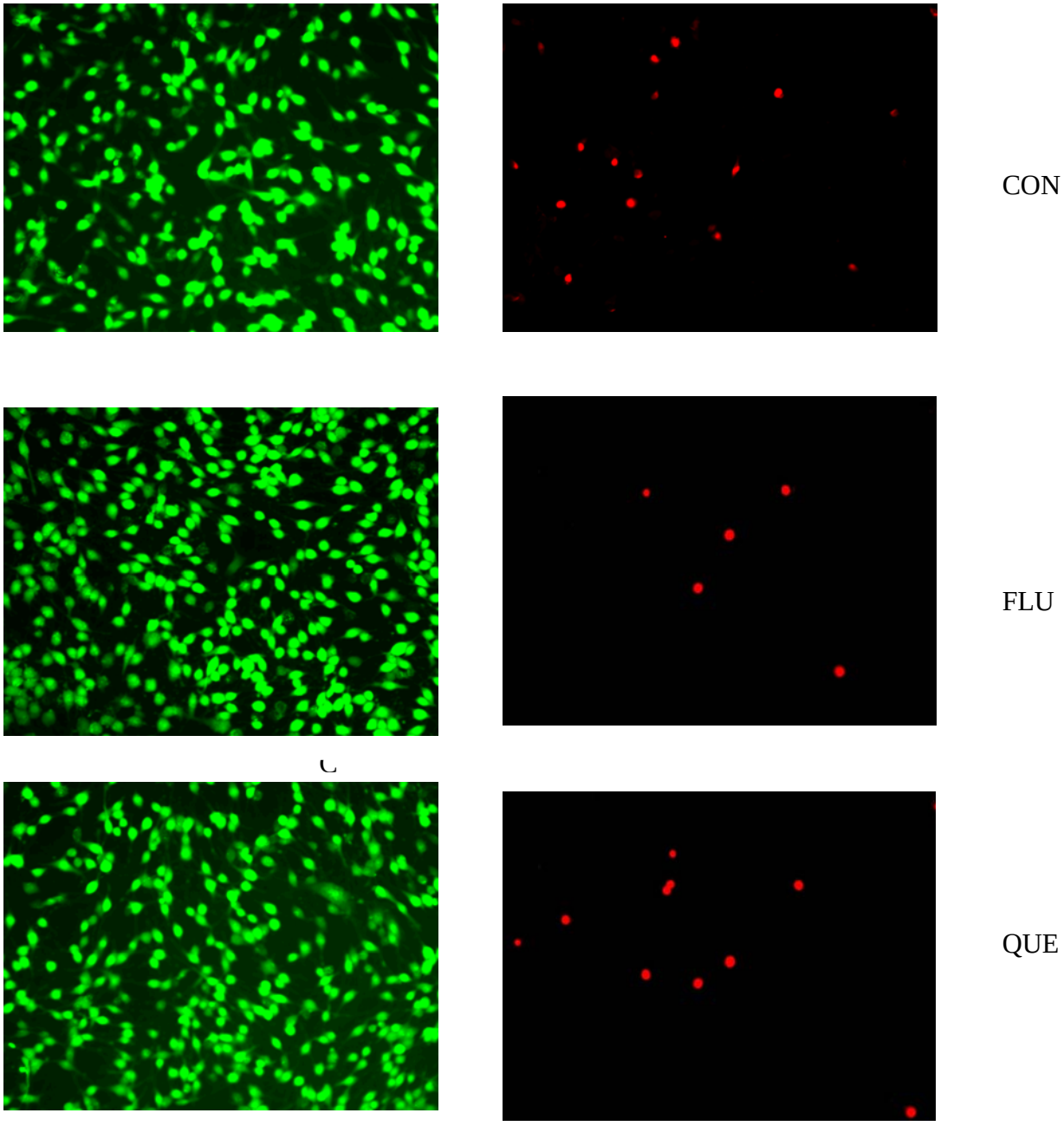
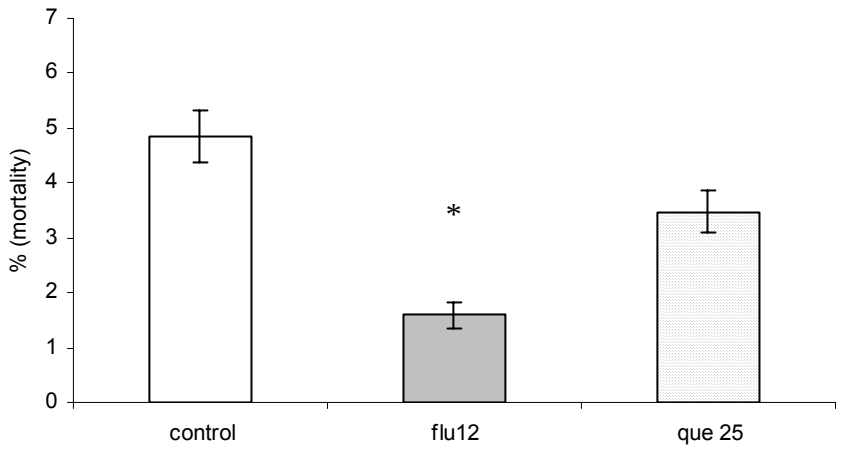


Fig 3.10 C6 cells stained by the Live - Dead Kit after 48 h treatment with quetiapine (25 μM) or fluoxetine (12.5 μM) under the serum starvation condition.

Magnification: 100×

Green color (left) :live cells Red color (right): dead cells

A



B

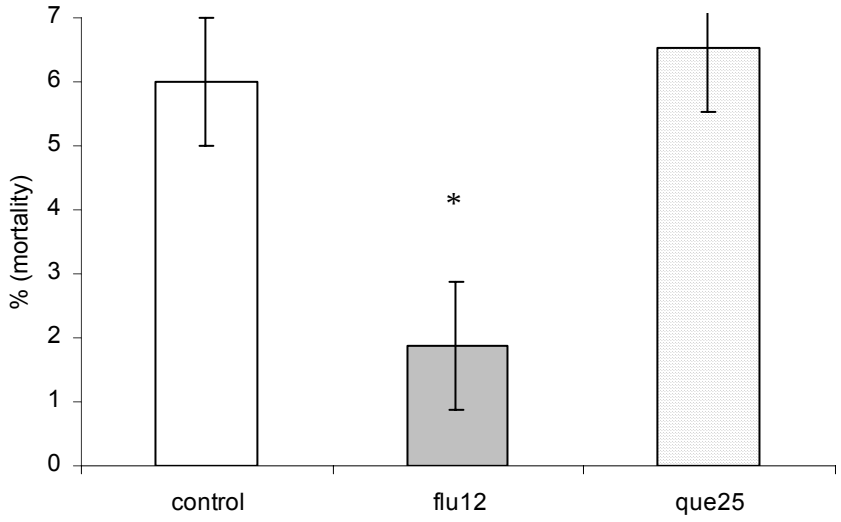


Fig 3.11 The percentage of dead C6 cells after 12 μM fluoxetine (flu12) or 25 μM quetiapine (que25) treatment at 24 h (A) and 48 h (B) under serum starvation. Values shown are mean±SEM (n=6). * $p<0.05$ compared to control.

3.4 The effect of fluoxetine and quetiapine on C6 cell morphology & GFAP content

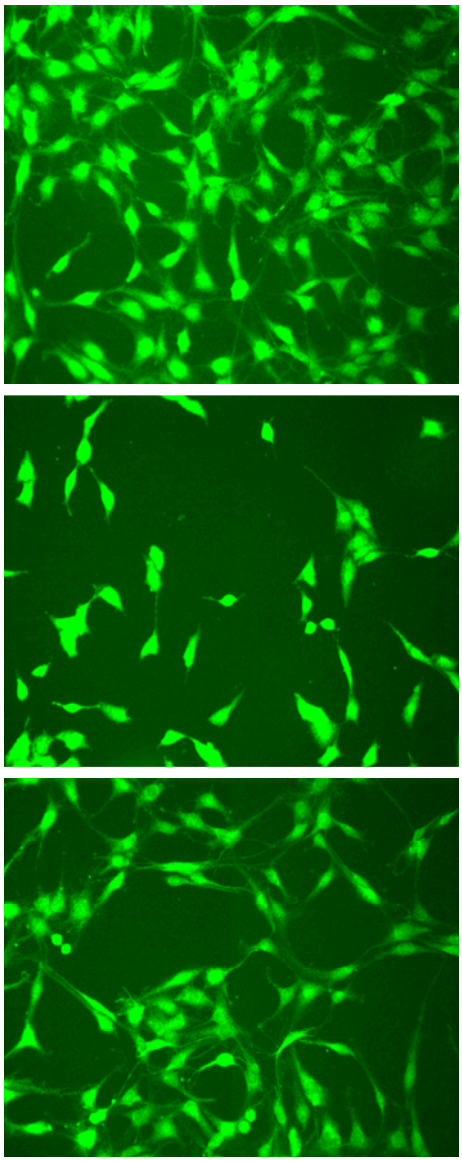
3.4.1 Microscopic observation of C6 cell morphology

After 24 h treatment, fluoxetine (12.5 μM) changed the morphology of C6 cells compared to control cells cultured under serum starvation conditions (see Fig 3.12). It can be seen that the processes of C6 cells in the fluoxetine group were relatively shorter compared to the control group. Quetiapine, however, did not appear to change the morphology of C6 cells. After 48 h treatment, the effect of fluoxetine on the morphology of the C6 cells was more prominent (see Fig 3.13). It can be seen that the processes of the C6 cells in the fluoxetine group were much shorter than those in the control group. However, there were no observable changes in the morphology of the C6 cells in the quetiapine treatment group compared to the control (see Fig 3.13).

3.4.2 Quantitative assessment of GFAP levels

At 24 h, fluoxetine increased the GFAP level of the C6 cells to 221±15% of control, while quetiapine had no significant effect on the level of GFAP (see Fig 3.14 A).

At 48 h, fluoxetine increased the GFAP level of the C6 cells to 189±30% of control, while quetiapine had no significant effect on the level of GFAP (see Fig 3.14 B).



A CON

B FLU

C QUE

Fig 3.12 Photomicrographs of C6 cells stained with a fluorescent marker for GFAP after 24 h treatment with quetiapine (25 μM) or fluoxetine (12.5 μM) under the serum starvation condition.
Magnification: 200×

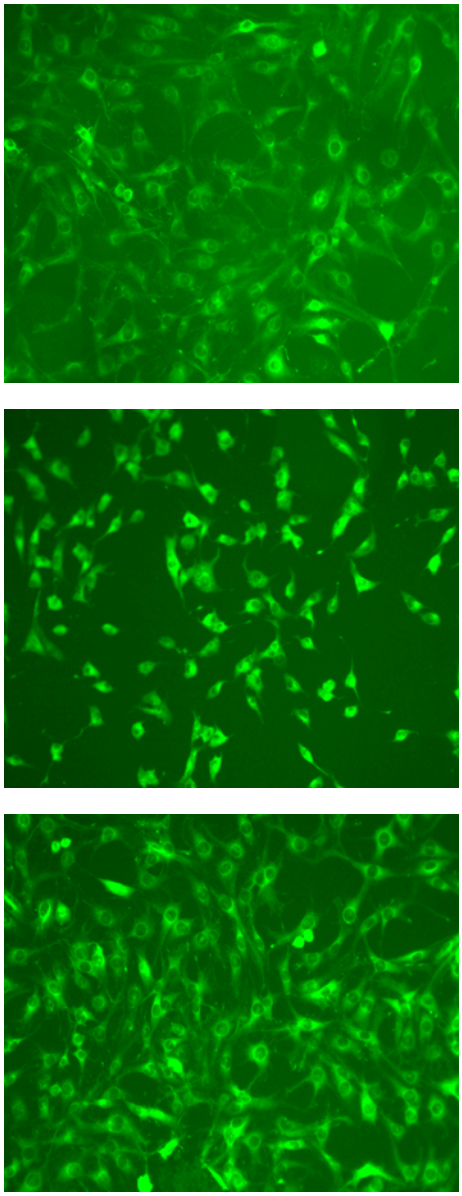


Fig 3.13 Photomicrographs of C6 cells of fluorescence stained with a fluorescent marker for GFAP after 48 h treatment with quetiapine (25 μM) or fluoxetine (12.5 μM) under the serum starvation condition.
Magnification: 200×

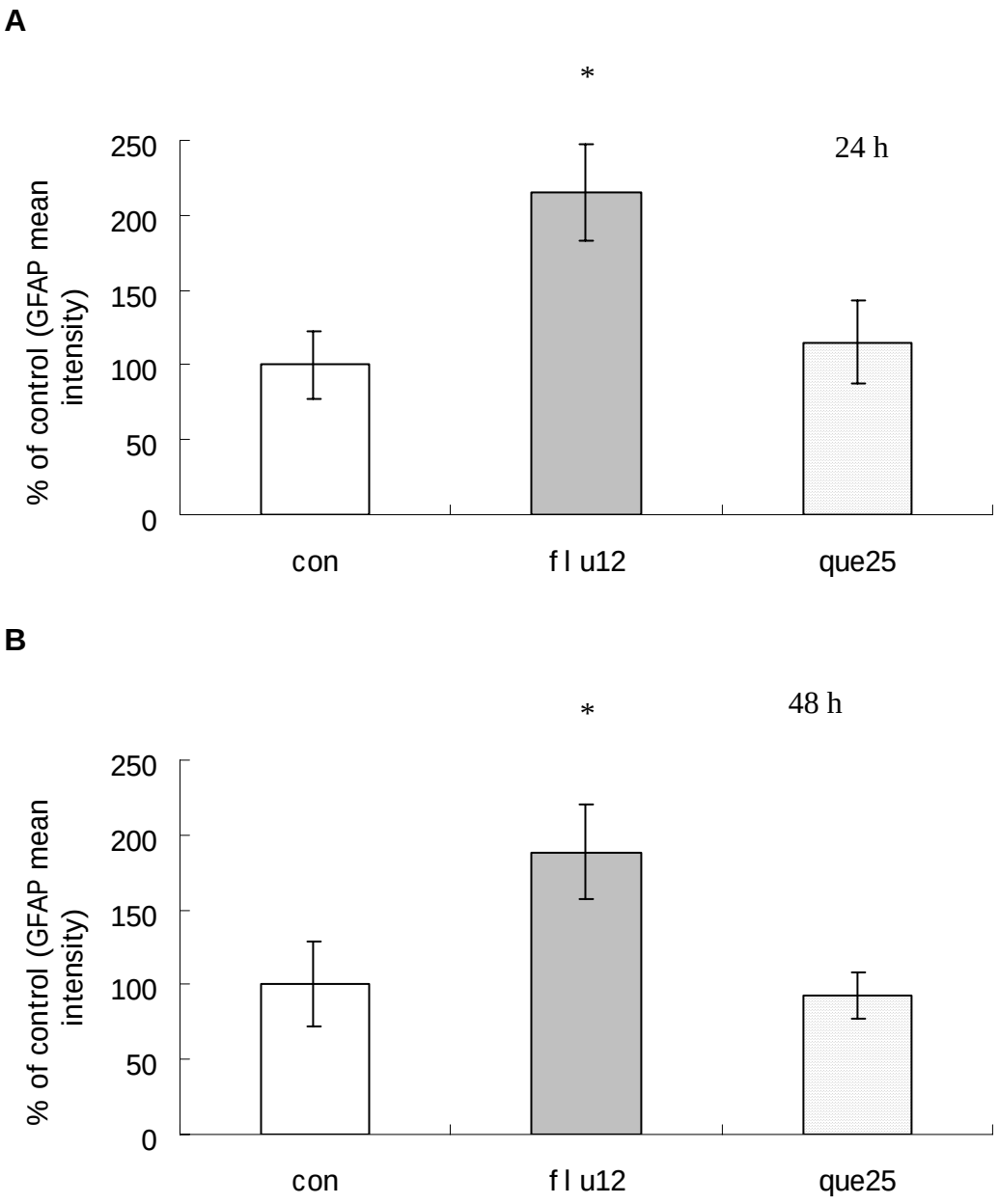


Fig 3.14 The intensity of GFAP immunofluorescent staining of C6 cells cultured under serum starvation conditions and treated with 12 μM fluoxetine (flu 12) or 25 μM quetiapine (que25) at 24 (A) and 48h (B). Values shown are mean±SEM (n=6). * $p<0.05$ compared to control.

3.5 The effects of quetiapine and fluoxetine on the release of GDNF

The relationship between the concentration of GDNF and absorption at 450 nM was linear from 15.6 pg/ml to 1000 pg/ml. The R^2 for the standard curve was always greater than 0.99 (see Fig 3.15).

In the initial experiments, the release of GDNF from the C6 cells cultured for 48 h under the serum present condition was found to be undetectable.

It can be seen, however, that under the serum starvation condition, both quetiapine and fluoxetine increased the release of GDNF from C6 cells after 48 h treatment in a dose-dependent manner (see Fig 3.16). Low concentrations of fluoxetine (1 and 6 μM) had no significant effect on the release of GDNF from C6 cells, but 12.5 and 25 μM fluoxetine increased the release of GDNF from C6 cells to 176±7% of control and 218±12% of control, respectively ($p<0.01$). Similarly, low concentrations of quetiapine (1, 6, and 12.5 μM) had no significant effect on the release of GDNF from C6 cells, while 25 μM quetiapine increased the release of GDNF from C6 cells to 195±20% of control ($p<0.01$) (see Fig 3.16).

The effect of fluoxetine (12.5 μM) on the release of GDNF was time-dependent. At early time points, the amount of GDNF release from C6 cells was quite low. The GDNF level after 12 h treatment was almost undetectable (see Fig 3.17). Fluoxetine (12.5 μM) had no significant effect on the release of GDNF at 24 h. After 48 h treatment, 12.5 μM fluoxetine increased the GDNF to 40±5 pg/ml ($p<0.01$) (see Fig 3.17).

Similarly, the effect of quetiapine (25 μM) on the release of GDNF was time dependent. The GDNF level at 12 h was almost undetectable (see Fig 3.17, Fig 3.18). Quetiapine (25 μM) had no significant effect on the release of GDNF at 24 h. After 48 h treatment, 25 μM quetiapine increased the GDNF to 60±7 pg/ml ($p<0.01$) (see Fig 3.18).

It was shown that the combination of a sub-effective concentration of quetiapine (6 μM) and sub-effective concentration of fluoxetine (6 μM) caused a greater release of GDNF from C6 cells compared to the control and to quetiapine alone ($p<0.01$ and $p<0.05$) (see Fig 3.19).

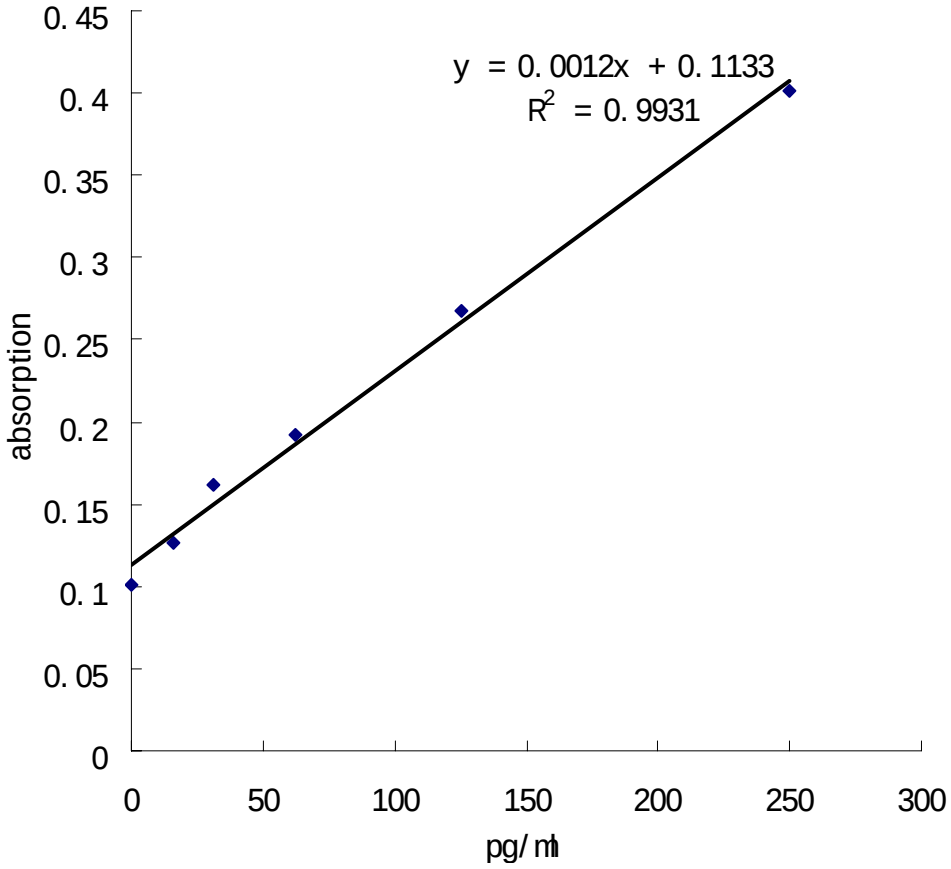


Fig 3.15 A typical GDNF ELISA standard curve.

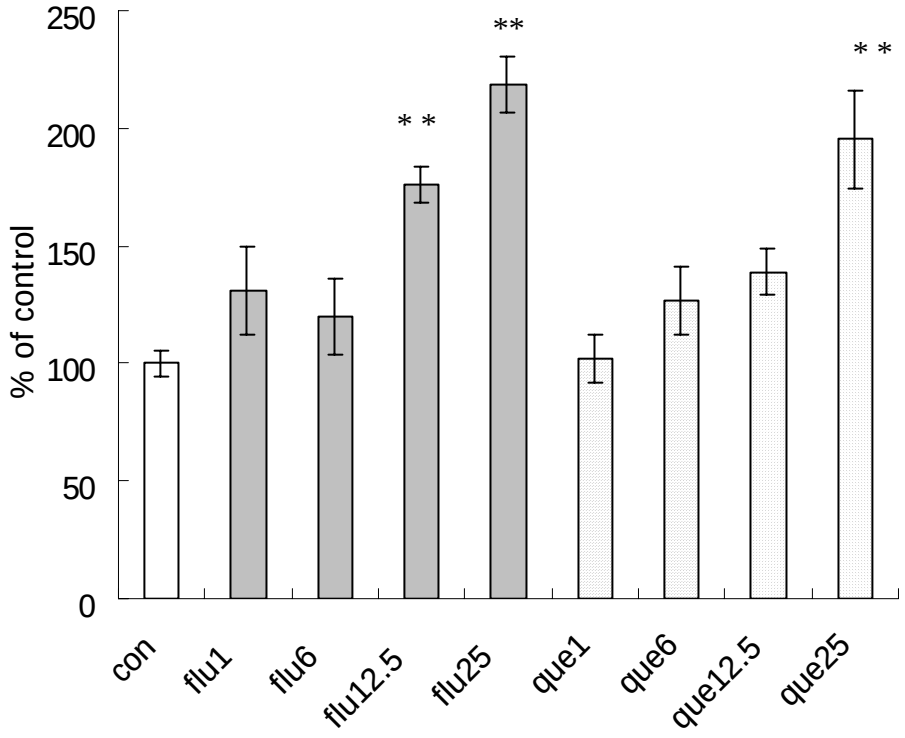


Fig 3.16 The effects of fluoxetine (flu concentration in μM) and quetiapine (que concentration in μM) on the release of GDNF from C6 cells cultured for 48 h under serum starvation conditions. Values shown are mean±SEM (n=6). * * $p<0.01$ compared to control.

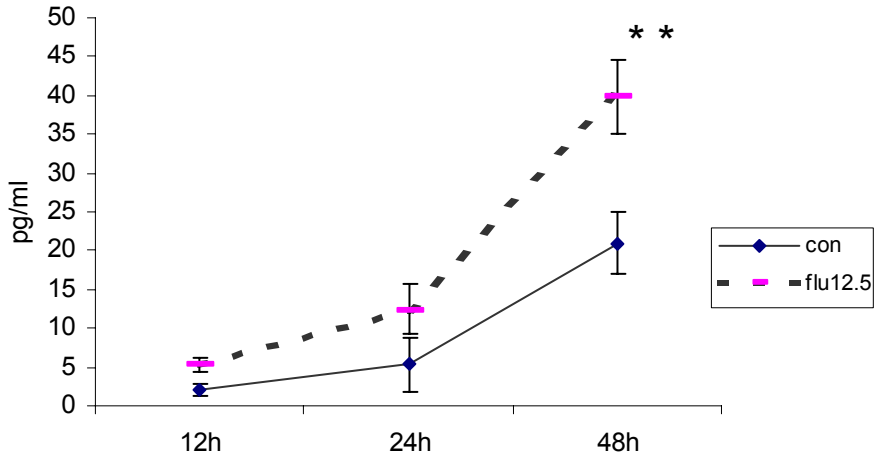


Fig 3.17 The effect of 12.5μM fluoxetine (flu 12.5) on the release of GDNF after culturing for different time periods. Values shown are mean±SEM (n=6). ** $p<0.01$ compared to control.

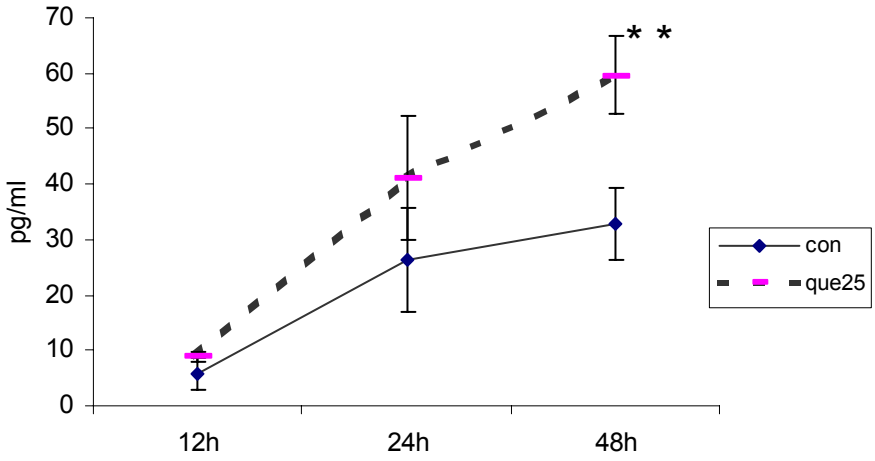


Fig 3.18 The effect of 25μM quetiapine (que 25) on the release of GDNF after culturing for different time periods. Values shown are mean±SEM (n=6). ** $p<0.01$ compared to control.

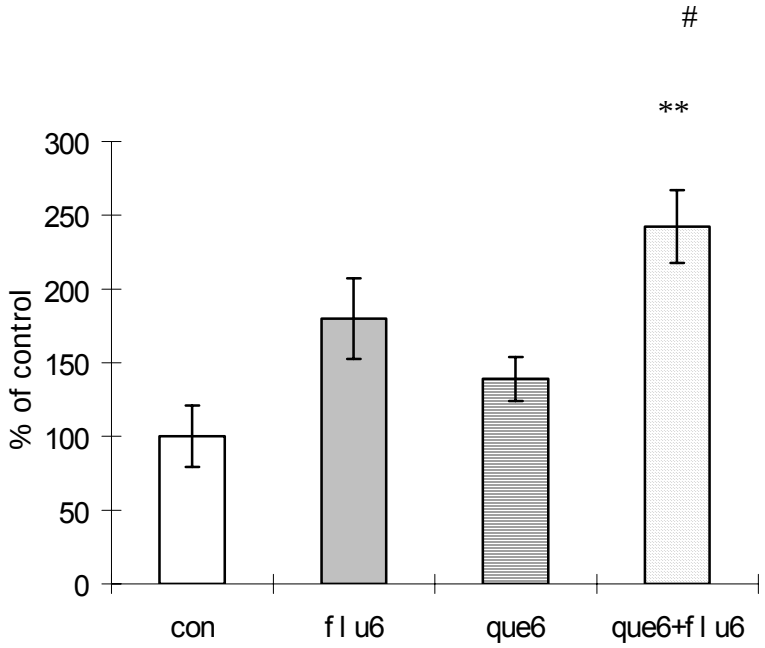


Fig 3.19 The effects of 6 μM quetiapine (que 6), 6 μM fluoxetine (flu 6) either alone or in combination on the release of GDNF. Values shown are mean ±SEM (n=9). # $p<0.05$ compared to quetiapine, ** $p<0.01$ compared to the control group.

Table 3.1 Summary of Results

		Serum present		Serum starvation	
		Fluoxetine	Quetiapine	Fluoxetine	Quetiapine
MTT conversion (cell number)	24h	25 μM ↓	No change	6, 12.5, 25 μM ↑	No change
	48h	25 μM↓	25 μM↓	12.5 ↑	25 μM↓
Proliferation	24h	12.5 μM↓	25 μM ↓	12.5 μM↓	25 μM ↓
Mortality	24h			12.5 μM↓	No change
	48h			12.5 μM↓	No change
Morphology	24h			Shorter processes	No change
	48h			Shorter processes	No change
GFAP	24h			12.5 μM ↑	No change
	48h			12.5 μM ↑	No change
GDNF	24h			Not significant	Not significant
	48h	Undetectable	Undetectable	12.5, 25 μM↑	25 μM↑

4 Discussion

4.1 The MTT conversion after quetiapine and fluoxetine treatment

4.1.1 C6 cells cultured under the serum present condition

The observation that fluoxetine decreased the MTT conversion in the serum present condition at 24 and 48 h (see Fig 3.1-3 and Table 3.1) could be interpreted as agreeing with other studies that showed several antidepressants, including fluoxetine (14.5-28.9 μM), induced apoptosis in C6 cells (Spanova et al., 1997; Slamon et al., 2001). An increase in apoptosis will decrease the viable cells; and thereby decrease the MTT conversion of C6 cells, which is a reflection of viable cell number.

A high concentration of quetiapine (25 μM) slightly decreased the MTT conversion only at 48 h (see Fig 3.2, 3.3 and Table 3.1). This is the first report that shows that quetiapine decreases the MTT conversion of C6 cells in culture. I think this may be a cytotoxic effect of quetiapine on C6 cells, because a high concentration of drugs might be toxic to cells. For example, 100 μM concentrations of the typical antipsychotics, such as fluphenazine, perphenazine and haloperidol, were cytotoxic to C6 cells, and it was concluded that this cytotoxicity was related to the activation of the sigma receptor by antipsychotics (Vilner and Bowen, 1993). The sigma receptor was originally proposed to be a subtype of the opioid receptor. Later it was demonstrated that sigma receptors are unique non-opioid, non-phencyclidine brain proteins. Two types of sigma receptor exist; the sigma-1 receptor and the sigma-2 receptor sigma-1 receptors

regulate glutamate NMDA receptor function and the release of neurotransmitters such as dopamine (Bourrie et al., 2004). If this effect of quetiapine is due to cytotoxicty, it cannot be attributed to the sigma receptor, since quetiapine has not been found to act at the sigma receptors.

4.1.2 C6 cells cultured under the serum starvation condition

This project focused on the serum starvation condition, because it was the condition used in previous experiments investigating GDNF release (Hisaoka et al., 2001; Shao et al., 2006). Because quetiapine and fluoxetine have been shown to increase GDNF release and since GDNF has trophic effects on C6 cells (Hisaoka et al., 2001; Shao et al., 2006; Suter-Crazzolara et al., 1996), an increase in the number of C6 cells after fluoxetine and quetiapine treatment was expected in my hypothesis. Fluoxetine (12.5 μM) did indeed increase the number of C6 cells (MTT conversion) from 24 to 48 h compared to control (serum starvation). By contrast, quetiapine (25 μM) decreased the number of C6 cells (MTT conversion) only at 48 h compared to control (see Fig 3.6 and Table 3.1). It can be concluded that fluoxetine, but not quetiapine, increased the cell number in the serum starvation condition.

This is the first report that antidepressants and antipsychotics affect the number of viable C6 cells cultured under serum starvation conditions. Neither Hisaoka nor Shao found any effect of antidepressants and antipsychotics on the number of viable C6 cells (Hisaoka et al., 2001; Shao et al., 2006). In Hisaoka's

experiment, 1.2×10^5 C6 cells were cultured in 12-well plates for 96 h, whereas in Shao's experiment, 4×10^5 C6 cells were cultured in 6-well plates for 96 h. In my experiments, 3×10^5 C6 cells were cultured in 6-well plates for 72 h. The density of C6 cells in my experiments was lower than those in Hisaoka's or Shao's experiments (Shao et al., 2006; Hisaoka et al., 2001). In the present project, the experimental conditions were modified, since the goals were not only to investigate the effects of fluoxetine and quetiapine on GDNF release from C6 cells, but also to investigate their effects on the proliferation and differentiation of C6 cells. C6 cells grow very fast even under serum starvation conditions. When the same cell density and culturing time period as those in Hisaoka's and Shao's experiments were tested, the C6 cells were almost confluent before drug treatment. So, the difference in the current findings compared to others may be due to the differences in culture conditions.

A study showed that GDNF is neurotrophic for glial cells (Suter-Crazzolara et al., 1996). Both quetiapine and fluoxetine increased the release of GDNF from C6 cells, but the increased number of C6 cells after fluoxetine treatment cannot be attributed solely to increased GDNF release, because quetiapine and fluoxetine had similar effects on the release of GDNF but opposite effects on the number of C6 cells in the serum starvation condition.

The MTT assay reflects mitochondrial function. An increase in formazan production may be due to an increase in the number of live cells and/or an increase in the metabolic activity of individual cells. To determine if the

increased MTT conversion after fluoxetine treatment was due to an increase in cell number caused by decreased cell death or the result of an increased overall metabolic activity of individual C6 cells, the following proliferation and cell death experiments were performed.

4.2 The effects of quetiapine and fluoxetine on proliferation of C6 cells

4.2.1 C6 cells cultured under serum present condition

In the serum present condition, both fluoxetine and quetiapine inhibited the proliferation of C6 cells measured after 24 h (see Fig 3.7). The direction of change of the effects of fluoxetine and quetiapine on proliferation matched their effects on the number of C6 cells (see Table 3.1). Fluoxetine decreased the number of C6 cells from 24 to 48 h, and quetiapine decreased the number of C6 cells after 48 h treatment. A decrease in proliferation may cause a decrease in the overall number of cells.

4.2.2 C6 cells cultured under serum starvation condition

In the serum starvation condition, both fluoxetine and quetiapine inhibited proliferation of C6 cell at 24 h (see fig 3.7). This was the first report of significant effects of quetiapine and fluoxetine on the proliferation of C6 cells in the serum starvation condition.

The direction of change of the effect of quetiapine on C6 cell proliferation matched its effect on the cell number (see Table 3.1). Inhibition of proliferation

at 24 h might be expected to decrease cell number, so this may explain the decreased number of C6 cells after quetiapine treatment at 48 h.

The inhibitory effect of fluoxetine on proliferation seems to be contradictory to its ability to increase the number of C6 cells (see Table 3.1). It could be expected that a decrease in proliferation leads to a decrease in cell number. However, a decrease in cell death might lead to a net increase in the number of viable cells. I speculated that fluoxetine might protect C6 cells from death caused by serum starvation. The increased number of C6 cells observed after fluoxetine treatment could be due to a decrease in cell death. So the following cell death experiments were performed to solve the apparent contradiction between the MTT conversion and cell proliferation results.

4.3 The effect of quetiapine and fluoxetine on the C6 cell death

As mentioned above, the cell death experiments were designed to solve the apparent contradiction between the MTT conversion and proliferation results after fluoxetine treatment in serum starvation condition; therefore, only the serum starvation condition was used in the cell death experiments.

Quetiapine had no effect on the number of dead C6 cells in the serum starvation conditions at 24 and 48 h (see Table 3.1). These results do not correlate with the results of the MTT conversion experiment of quetiapine at 48 h (see Table 3.1). Thus, the decrease in the number of C6 cells caused by quetiapine after 48 h treatment cannot be attributed to the increase in cell death.

Fluoxetine decreased the number of dead C6 cells in the serum starvation condition (see Table 3.1). This means that fluoxetine protected C6 cells from death caused by the serum starvation. The protective effect of fluoxetine for C6 cells in the serum starvation condition seems contradictory to some literature, which found that fluoxetine can induce apoptosis (Serafeim et al., 2002; Levkovitz et al., 2005). The different results may be due to different conditions. It is possible that fluoxetine decreases the cell death of C6 cells only in serum starvation conditions, i.e., a protective effect. A study found that fluoxetine can prevent apoptosis in dentate gyrus of maternally separated rats (Fatemi et al., 2004), which is a model of stress. It was also that fluoxetine protected against staurosporine-induced apoptotic cell death (Nahon et al., 2005), while fluoxetine protected PC12 cells from cell death induced by hydrogen peroxide (Kolla et al., 2005). It is possible that the protective effect of fluoxetine occurs only under stressed conditions. In fact, antidepressants do not enhance the mood of the normal person; they can only improve the mood of patients with depression (Emette, 1985). So the protective effect of fluoxetine is in accordance with clinical results.

Fluoxetine decreased the percentage of dead C6 cells from 5 to 2% in the serum starvation condition at 24 h (see Fig 3.11); however, fluoxetine (12.5 μM) increased the number of C6 cells to 131% of control. It seems unlikely that the large increase in the number of C6 cells can be totally attributed to the relatively small decrease in the percentage of dead cells. Another possibility is that

fluoxetine changed the metabolic activity of individual C6 cells or of the enzyme that converts MTT; so experiments were designed to investigate the effects of fluoxetine and quetiapine on the morphology and GFAP content of C6 cells under serum starvation conditions.

4.4 The effects of quetiapine and fluoxetine on the morphology and GFAP content of C6 cells

Quetiapine and fluoxetine had different effects on the morphology and GFAP level of C6 cells (see Fig 3.14 and Table 3.1). Fluoxetine induced a morphological change in C6 cells and increased GFAP level in the serum starvation conditions. Quetiapine had no significant effects on the morphology and GFAP level of C6 cells. No previous report on the effect of fluoxetine on the morphology of C6 cells and its effect on the level of GFAP of C6 cells was found.

In the study of differentiation of C6 cells, two parameters are often used. One is morphological change. The morphological changes reported by different laboratories vary somewhat, and this is thought due to differences in the culture conditions (Zimmer and Van Eldik, 1989). For example, a morphological change from flat to spindle shape after differentiation was reported; and a morphological change from bipolar to multipolar shape was also reported (Zimmer and Van Eldik, 1989; Takanaga et al., 2004). Even similar culture conditions cannot ensure similar morphological changes after induction of differentiation; for instance, the morphology of C6 cells are quite different between early passages

and late passages (Goya et al., 1996). The second parameter used to study differentiation is to measure the levels of GFAP, a marker for mature astrocytes. Increased GFAP levels indicate differentiation of C6 cells into astrocytes (Zimmer and Van Eldik, 1989).

In my experiments, it was found that the processes of C6 cells after fluoxetine treatment were relatively shorter than the control (see fig 3.12-3). Furthermore, an increase in GFAP content after fluoxetine treatment was observed (see fig 3.14). The observed morphological change of C6 cells and upregulation of GFAP after fluoxetine treatment indicate differentiation of C6 cells. The individual differentiated cells may have an increased metabolic activity compared to control, which might explain the increase in MTT conversion of C6 cells after fluoxetine treatment in the serum starvation condition, and as explained in Section 4.3 could not be totally attributed to the decrease in the cell death.

There is evidence that patients with depression have lower levels of GFAP and a decreased astrocyte number (Rajkowska et al., 1999). If these are the underlying factors of depression, it is possible that the protective effect of fluoxetine for C6 cells (which are a model for astrocytes) and the effect of fluoxetine on upregulation of GFAP may play a role in the mechanisms of antidepressants. This, however, needs to be confirmed by direct evidence using primary cell culture studies and animal models of depression.

4.5 The release of GDNF after quetiapine and fluoxetine treatment

The current results demonstrated that both fluoxetine and quetiapine can increase the release of GDNF from C6 cells in the serum starvation condition in a time- and dose-dependent manner (see fig 3.16-8 and table 3.1). These results confirmed previous results showing that both typical and atypical antipsychotics could induce the release of GDNF from C6 cells (Shao et al., 2006).

The absolute value of GDNF measured after quetiapine treatment was lower than that in Shao's experiment (around 200 pg/ml vs. around 60 pg/ml); this may be due to differences in the cell density.

The current results also confirmed previous results which found that several kinds of antidepressants, including fluoxetine, could increase the release of GDNF from C6 cells cultured in serum starvation conditions after 48 h (Hisaoka et al., 2001). They did not find that haloperidol could modulate the release of GDNF, but only 1 μM haloperidol was used in their experiments. In Shao's experiment (Shao et al., 2006), 12.5 and 25 μM haloperidol were found to increase the release of GDNF, while concentrations of haloperidol lower than 12.5 μM had no such effect. It is clear that the release of GDNF by haloperidol is dose-dependent.

The fact that both antidepressants and antipsychotics can increase the release of GDNF may indicate a possible common mechanism in the treatment of depression. This may be more relevant for atypical antipsychotics, because

atypical antipsychotics can be used clinically for the treatment of depression (Blier and Szabo, 2005; Nemeroff, 2005). It is possible that atypical antipsychotics may share some similarity in the modulation of growth factors with antidepressants. It was found that both antidepressant and atypical antipsychotics can increase the level of BDNF in animal models of depression (Manji et al., 2000). The current experiment and those reported in the literature demonstrated that both antidepressants and antipsychotics could increase the release of GDNF from C6 cells cultured in serum starvation condition.

Dopamine, several mixed dopamine agonists and dopamine D1 receptor agonists can increase GDNF secretion from primary cultures of rodent astrocytes and mesencephalic cells; however, a dopamine D2 receptor agonist decreased the secretion of GDNF (Ohta et al., 2000; Guo et al., 2002; Ohta et al., 2003). It is well known that antipsychotic drugs are dopamine D2 receptor blockers. So blockade of dopamine D2 receptor may be involved in the effect of antipsychotic drugs on GDNF. However, this is only a speculation, because there are no reports on the presence of dopamine D2 receptors on C6 cells, so it is too early to attribute the effect of antipsychotics on the release of GDNF to D2 receptor blockade.

Neither previous work (Shao et al., 2006) nor the present experiments examined the mechanism of antipsychotics in modulation of the release of GDNF from C6 cells; more experiments are needed to elucidate the mechanism of the effect of antipsychotics on the release of GDNF. There have, however,

been several papers that have investigated the mechanism of the release of GDNF by antidepressant drugs. One such study showed that 100 μM serotonin, but neither dopamine nor noradrenaline, increased secretion of GDNF from rat C6 cells (Hisaoka et al., 2004). This effect was blocked by inhibition of the MAP kinase signaling pathway, but not by inhibitors of protein kinase A or protein kinase C. It was found that U0126 (a mitogen-activated protein kinase inhibitor) can partially block the secretion of GDNF in C6 cells induced by antidepressants (Hisaoka et al., 2004). It is well known that antidepressants can increase the available concentration of serotonin. So it is possible that antidepressants increase the secretion of GDNF in C6 cells via MEK/MAPK signaling pathway.

Sub-effective concentrations of quetiapine and fluoxetine were chosen to examine the potential synergistic effect between fluoxetine and quetiapine, because it has been shown that patients benefit from combining low doses of these drugs (Bowden, 2004). GDNF levels following the combined treatment were greater than single use of quetiapine and the control group. The combination of quetiapine and fluoxetine had an apparent additive effect in the modulation of GDNF in C6 cells.

The effect of combining antipsychotic and antidepressant drugs has been studied in the modulation of BDNF. The antidepressant, venlafaxine, and atypical antipsychotic drug, quetiapine, had a synergistic effect in the upregulation of BDNF in stressed depression animal model (Chen et al., 2005).

The ability of quetiapine and fluoxetine to modulate GDNF release may

underlie their benefit in treating depression and schizophrenia.

5 Conclusions

From these experiments, the following conclusions can be made.

1. High concentrations (25 μM) of fluoxetine and quetiapine decreased the number of C6 cells under the serum present condition and both drugs inhibited the proliferation of C6 cells.

2. Fluoxetine had a protective effect on the C6 cells under serum starvation, and affected the differentiation of C6 cells; this implies that fluoxetine may protect glial cells in vivo and affect their differentiation.

3. A high concentration of quetiapine decreased the number of C6 cells and inhibited the proliferation under serum starvation; even though it increased the release of GDNF from C6 cells as did fluoxetine.

4. Both quetiapine and fluoxetine increased the release of GDNF from C6 cells under serum starvation. The combination of quetiapine and fluoxetine had an apparent additive effect in the modulation of GDNF release.

5. These effects on proliferation & GDNF release may underlie the benefit observed with these drugs in treating depression and schizophrenia.

6 Future work

Because the mechanism of GDNF secretion induced by antipsychotics is still not clear; in the future this question needs to be addressed. Drugs that

block signaling pathways, such as U0126 (a MAPK kinase inhibitor) and SF1126 (a PI3K inhibitor), can be used to address this question.

Primary astrocytes can also be used to investigate the effects of antipsychotics and antidepressants on the proliferation, differentiation and the release of GDNF in primary astrocytes.

We can also study the effect of antidepressants or antipsychotics on glial cells in depression models, to see if there is a protective effect of fluoxetine for glial cells, because glial cell loss may be a cellular mechanism of depression.

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